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(54) Title: HUMAN CHEMOKINE AND ITS USE FOR DETECTION AND TREATMENT OF BREAST DISEASE (57) Abstract <p>An isolated chemokine is disclosed. The isolated chemokine is expressed preferentially in breast tissue or can be detected in breast milk. It includes from about 100 to about 132 amino acids, has a deduced molecular weight of from about 10 to about 16 kDa, and has a deduced isoelectric point of from about pH 10.1 to about pH 10.7. Antibodies and binding portions thereof recognizing the subject chemokine and peptides which include the antigenic portions of the subject chemokines are described. DNA molecules which encode the subject chemokines as well as nucleic acid molecules which, under stringent conditions, hybridize to nucleic acid molecules encoding the subject chemokines or to a complement thereof are also disclosed. The chemokines, peptides, antibodies and binding portions thereof, and nucleic acid molecules can be used to detect and treat breast disease, such as inflammations, infections, mastitis, benign cystitis, benign hyperplasias, cancer and other malignancies as well as other pathological states of the mammary gland.</p>			

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HUMAN CHEMOKINE AND ITS USE FOR DETECTION AND TREATMENT OF BREAST DISEASE

This application claims the benefit of U.S. Provisional Patent Application
Serial No. 60/071,899, filed January 20, 1998, and U.S. Provisional Patent Application
5 Serial no. 60/092,155, filed July 9, 1998, which are hereby incorporated by reference.

FIELD OF THE INVENTION

The present invention relates to the detection and treatment of breast
disease.

BACKGROUND OF THE INVENTION

10 Breast cancer is one of the largest classes of malignant disease in women.
However, breast cancer presents inherent difficulties in regard to the ease with which it is
detected and diagnosed. This is in contrast to detection of some other common cancers,
including skin and cervical cancers, the latter of which is based on cytomorphicologic
screening techniques.

15 Early detection of breast cancer represents a compelling goal in oncology.
Although techniques such as computerized tomography, mammography, and magnetic
resonance imaging have greatly improved tumor surveillance over the past decade, there
still remains a need for serologic and other blood-based assays.

Serologic assays are easily performed, inexpensive, and
20 analytically-sensitive and can be serially run over time with relative ease. The essence of
breast cancer screening, using tumor marker detection, is to efficiently identify a group of
higher-risk individuals from within a large population. Thereafter, confirmatory testing is
implemented to establish a diagnosis of malignancy.

There are several classifications of tumor markers possible, based upon the
25 structure or biological function of the marker. Tumor marker classifications include
tissue specific antigens (e.g., PSA, NSE, PAP, calcitonin, HCG), major histocompatibility
complex ("MHC") antigens, viral antigens (e.g., HTLV-I gag protein), oncogene products
(e.g., c-HER-2/Neu), oncofetal markers (e.g., CEA, AFP), hormones (e.g., thyroid
hormones), enzymes (e.g., telomerase, galactosyltransferase), and altered
30 glycoproteins/glycolipids (e.g., polymorphic epithelial mucins). It should be noted that
these classification schemes are imprecise and contain redundancies. For example,
calcitonin is an important serological marker for medullary carcinoma of the thyroid and
may be classified not only as a hormone but also as a tissue specific protein of the

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thyroid. Likewise, PSA, HCG, thyroid hormones, PAP, and NSE are tissue specific proteins and also exhibit enzymatic or hormonal activities. Generally, tumor markers providing high clinical utility reside in the broadly defined tissue specific class. This class of tumor markers contains enzymes, isoenzymes, hormones, growth factors, and
5 other molecules with biologic activity.

The importance of a tumor marker's being tissue specific is illustrated by one of the best known tumor antigens, carcinoembryonic antigen ("CEA"). When first discovered, CEA was thought to be specific to cancers of the digestive system. However, CEA has since been detected in normal adults as well as in patients with benign liver
10 disease, such as alcoholic hepatitis or biliary obstruction. Because of the overall lack of specificity and sensitivity, there being no threshold difference in CEA levels that serves to separate benign from malignant conditions, CEA cannot be used in a general diagnostic test. Instead, it is principally used to monitor a patient's response to treatment.

To be useful in serologic assays, a tumor marker should be one that is
15 released into the bloodstream as a circulating marker. Circulating antigens are now known to exist in breast cancer. Breast tissue markers, such as casein (Franchimont et al., Cancer, 39:2806-2812 (1977)) and α -lactalbumin (Kleinberg et al., Science, 190:276-278 (1975)) and purported cancer markers, such as glycosyl transferases (Ip et al., Cancer Res., 38:723-728 (1978) and Dao et al., J. Natl. Cancer Inst., 65:529-534 (1980)),
20 glycolipids (Kloppel et al., Proc. Natl. Acad. Sci. USA, 74:3011-3013 (1977)), and phospholipids (Skipski et al., Proc. Soc. Exp. Biol. Med., 136:1261-1264 (1971)) have all been used in various diagnostic techniques for breast cancer but have not gained widespread acceptance as breast cancer markers. More recently, circulating human mammary epithelial antigens have been proposed as specific markers for breast cancer
25 (Ceriani et al., Proc. Natl. Acad. Sci. USA, 79:5420-5424 (1982)). Burchell et al., Int. J. Cancer, 34:763-768 (1984) describes monoclonal antibodies which detect high molecular weight mucin-like antigens elevated in patient serum. Hayes, J. Clin. Invest., 75:1671-1678 (1985) also describes a monoclonal antibody that recognizes a high molecular weight mammary epithelial antigen present in elevated amounts in the plasma
30 of breast cancer patients. See also Papsidero et al., Cancer Res., 44:4653-4657 (1984) and Taylor-Papadimitriou et al., Int. J. Cancer, 28:17-28 (1981). Other breast tissue specific proteins or markers include alpha, beta, and kappa caseins, alpha-lactalbumin, lactoferrin, and selected epithelial membrane antigens. These are described in Cohen et

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al., Cancer, 60:1294-1298 (1987); Bartkova, Eur. J. Cancer Clin. Oncol., 23:1557-1563 (1987); Weir et al., Cancer Detect. Prev., 4:193-204 (1981); de Almeida et al., Breast Cancer Res. Treat., 21:201-210 (1992); Skilton et al., Tumor Biol., 11:20-38 (1990); Earl et al., Cancer Res., 49:6070-6076 (1989); Barry et al., Amer. J. Clin. Path., 82:582-585 (1984); and Watson et al., Cancer Res., 56:860-865 (1996). None of these previously described antigens has been used as a basis for a widely accepted breast cancer clinical assay.

There have also been several attempts to develop improved methods of breast cancer detection and diagnosis based on oncogene mutations, gene amplification, and loss of heterozygosity in invasive breast cancer. These methods have not gained wide acceptance.

Despite the use of mammography and the development of some breast tissue specific markers, there still remains a need for simple and rapid methods for detecting breast cancer. The present invention is directed to meeting this need.

15

SUMMARY OF THE INVENTION

One aspect of the present invention relates to an isolated chemokine that is preferentially expressed in breast tissue or which can be detected in breast milk. The isolated chemokine includes about from about 100 to about 132 amino acids, has a deduced molecular weight of from about 10 to about 16 kDa, and has a deduced isoionic point of from about pH 10.1 to about pH 10.7.

The present invention also relates to peptides having an amino acid sequence corresponding to an antigenic portion of the subject chemokine, to antibodies which recognize this chemokine, and to isolated nucleic acid molecules which encode this chemokine.

The present invention also relates to an isolated nucleic acid molecule which, under stringent conditions, hybridizes to a nucleic acid molecule encoding a chemokine of the present invention or to a complement thereof.

In another aspect thereof, the present invention relates to an isolated nucleic acid molecule which encodes for a chemokine of the present invention.

The present invention also relates to a method for detecting breast disease in a patient. A sample of tissue or body fluid from the patient is contacted with a nucleic

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acid primer which, under stringent conditions, hybridizes to a nucleic acid molecule encoding a chemokine of the present invention or to a complement thereof. The sample of tissue or body fluid from the patient in contact with the nucleic acid primer is treated under conditions effective to amplify breast tissue specific nucleic acid molecules. The method further includes detecting the breast tissue specific nucleic acid molecules.

The present invention also relates to another method of detecting breast disease in a patient. In this method, a sample of tissue or body fluid from the patient is contacted with a nucleic acid probe under conditions effective to permit formation of a hybridization complex between the probe and breast tissue specific nucleic acid molecules. The nucleic acid probe is one which, under stringent conditions, hybridizes to a nucleic acid molecule encoding a chemokine of the present invention or to a complement thereof. The method further includes detecting the hybridization complex.

The present invention also relates to yet another method of detecting breast disease in a patient. The method includes providing an antibody or binding portion thereof which recognizes a chemokine of the present invention. The antibody or binding portion thereof is contacted with a liquid or tissue sample from the patient under conditions effective to permit binding of the antibody or binding portion thereof to the chemokine in the liquid or tissue sample. The method further includes detecting presence of antibody or binding portion thereof bound to the chemokine in the liquid or tissue sample.

The present invention, in another aspect thereof, relates to a method of treating breast disease in a patient. The method includes administering to the patient an effective amount of an antibody or binding portion thereof which recognizes a chemokine of the present invention.

The present invention also relates to another method of treating breast disease in a patient. The method includes administering to the patient an effective amount of a peptide which binds to a cellular receptor for a chemokine of the present invention.

The present invention also relates to a method of vaccinating a patient against breast disease. The method includes administering to the patient an effective amount of an antigenic portion of a chemokine of the present invention.

The chemokines, peptides, antibodies, and nucleic acid molecules of the present invention are useful in the early detection of various pathological states of the

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mammary gland, such as inflammations, infections, benign hyperplasias, and malignancies. In particular, they can be used in the early detection of breast cancer as well as for monitoring the presence or absence of metastatic breast cancer cells in a patient's tissues and fluids, such as blood, lymph nodes, bone marrow, and other sites of disease dissemination. They can also be used to stage patients with breast cancer and to assess the effects of conventional breast cancer therapies. Furthermore, the chemokines, peptides, and antibodies of the present invention can be used to treat or prevent breast disease.

10

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 is a series of aligned amino acid sequences of various members of the CC chemokine family and the amino acid sequence of a chemokine of the present invention.

15

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to an isolated chemokine that is preferentially expressed in breast tissue or that is detectable in breast milk. Chemokine, as used herein, is meant to include proteins which are proinflammatory cytokines that are chemoattractants and activators of specific types of leukocytes. Further details with respect to chemokine activity can be found, for example, in U.S. Patent No. 5,688,927 to Godiska et al. and Baggiolini et al., Advances in Immunology, 55:97-179 (1994), which are hereby incorporated by reference. The chemokine may include a leader sequence, typically about 22 amino acids in length, or, alternatively, the leader sequence can be cleaved from the chemokine. The isolated chemokine preferably includes from about 100 to 132 amino acids, more preferably, from about 105 to about 127 amino acids, and, most preferably, about 105 or 127 amino acids. The deduced molecular weight of the chemokine of the present invention is preferably from about 10 to about 16 kDa, more preferably, from about 12 kDa to about 14 kDa, and preferably has a deduced isoelectric point of from about pH 10.1 to about pH 10.7, more preferably about 10.4.

30

As indicated above, the chemokine of the present invention is preferentially expressed in breast tissue. That is, more chemokine of the present invention is expressed in breast tissue than in any other tissue in the body. More

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preferably, the chemokine of the present invention is expressed substantially exclusively or exclusively in breast tissue. That is, substantially all of the chemokine of the present invention is expressed in breast tissue. In addition or alternatively to being preferentially expressed in breast tissue, the chemokine of the present invention can be detected in
 5 breast milk, such as by using conventional protein detection methods.

One particularly preferred chemokine of the present invention has an amino acid sequence corresponding to SEQ. ID. No. 1, as follows:

10 MQQRGLAIVALAVCAALHASEAILPIASSCCTEVSHHISRLLERVNMCRIQRAD
 GDCDLAAVILHVKRXRICVSPHNHTVKQWMKVQAAXKNGKGNVCHRKKHHG
 KRNSNRAHQGKHETYGHKTPY

As indicated above, chemokine, as used herein, can include a leader sequence, or, alternatively, all or part of the leader sequence may be removed. In SEQ.
 15 ID. No. 1, approximately the first 22 amino acids represents the leader sequence. Thus, chemokines of the present invention can also have an amino acid sequence corresponding to, for example, SEQ. ID. No. 2, as follows:

20 LPIASSCCTEVSHHISRLLERVNMCRIQRADGDCDLAAVILHVKRXRICVSPHNH
 TVKQWMKVQAAXKNGKGNVCHRKKHHGKRNSNRAHQGKHETYGHKTPY

The chemokine of the present invention is isolated (i.e., substantially free of the biological materials with which it is naturally found). In many applications, it is desirable that the chemokine of the present invention be purified (i.e., substantially free of
 25 all other biological materials). The chemokines of the present invention can be in monomer form, or they can be associated with other chemokines, such as in the form of dimers.

The present invention also relates to peptides which include an amino acid sequence corresponding to an antigenic portion of a chemokine of the present invention.
 30 In general, the size of the peptide antigen is not believed to be particularly crucial, so long as it is at least large enough to carry the antigenic core sequence or sequences. Generally, the smallest useful antigenic sequence is on the order or about six amino acids in length. However, the size of the antigen may be larger where desired, so long as it contains a basic antigenic core sequence.

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Accordingly, through the use of computerized peptide sequence analysis program (DNASTar Software, DNASTar, Inc., Madison, Wisconsin), the portions of the peptide can be identified that are believed to constitute antigenic sequences which include particular epitopes of the protein. More particularly, antigenic portions of a chemokine of the present invention can be identified by hydrophathy analysis, such as that described in 5 Kyte et al., "A Simple Method for Displaying the Hydrophathic Character of a Protein," J. Mol. Biol., 157:105-132 (1982), which is hereby incorporated by reference.

Synthesis of peptides which include an antigenic epitope within their sequence, are readily achieved using conventional synthetic techniques such as the solid 10 phase method (e.g., through the use of commercially available peptide synthesizer such as an Applied Biosystems Model 430A Peptide Synthesizer). Peptides synthesized in this manner may then be aliquoted in predetermined amounts and stored in conventional manners, such as in aqueous solutions or, even more preferably, in a powder or lyophilized state pending use.

15 Particularly preferred peptides of the present invention are those which include amino acid sequences corresponding to TEVSHHISRLLERVNMC (SEQ. ID. No. 3), KNGKGNVCHRKKHHGK (SEQ. ID. No. 4), and NSNRAHQGKHETYGHKTPY (SEQ. ID. No. 5).

As described below, the chemokines or peptides of the present invention 20 can be used to raise antibodies that recognize chemokines of the present invention. The chemokines and peptides of the present invention can also be administered alone or in combination with a pharmaceutically-acceptable carrier to patients, as a vaccine, for preventing breast disease.

The present invention also relates to antibodies and binding portions 25 thereof which recognize a chemokine according to the present invention. Preferably, the antibody or binding portion thereof also recognizes particular antigenic portions of the subject chemokine, such as peptides having amino acid sequences corresponding to SEQ. ID. No. 3, SEQ. ID. No. 4, and SEQ. ID. No. 5.

The antibodies and binding portions thereof can be used to detect breast 30 disease in a patient. As used herein, breast disease is meant to include various pathological states of the mammary gland, such as inflammations, infections, mastitis, benign cystitis, benign hyperplasias, and cancer and other malignancies. Detection of breast disease involves providing an antibody or binding portion thereof which recognizes

a chemokine of the present invention. The antibody or binding portion thereof is contacted with a tissue or fluid sample from the patient under conditions effective to permit binding of the antibody or binding portion thereof to chemokine that is present in the tissue or fluid sample to form a complex. The presence of a chemokine of the present invention in the tissue or fluid sample is detected by detecting the complex.

Such contacting can be carried out *in vivo* in a living patient. In this embodiment of the present invention, the antibody or binding portion thereof is administered (e.g., orally or parenterally) to the patient under conditions effective to permit binding of the antibody or binding portion thereof to the chemokine of the present invention in the *in vivo* tissue or fluid sample. Using this method, patients can be screened for breast diseases associated with the presence of chemokines of the present invention. Alternatively, the method can be used to identify the recurrence of such diseases, particularly when the disease is localized in a particular biological material of the patient. For example, recurrence of breast disease in a patient's breast tissue can be detected by administering a short range radiolabeled antibody to the patient and then imaging the breast using conventional radiation imaging techniques to detect the presence of the radiolabel and, therefore, a concentration of a chemokine of the present invention, within the breast. Similarly, by imaging other portions of the patient's body (e.g., lymph nodes), the method can be used to determine whether breast disease (e.g., breast cancer) has spread to other tissues of the body.

Alternatively, the contacting step can be carried out *in vitro*. For example, the tissue or fluid sample can be a tissue specimen (e.g., cells or tissue sections, preferably preserved by freezing or embedding in paraffin, from the breast, lymph nodes, bone marrow, or other sites of disease dissemination). Alternatively, the tissue or fluid sample can be a fluid specimen (e.g., urine, serum, lymph fluid, and anticoagulated whole blood cells) removed from the patient.

The antibodies and binding portions thereof of the present invention can also be used to treat breast disease, for example, by ablating or killing diseased breast tissue cells. The process involves providing an antibody or binding portions thereof which recognizes a chemokine of the present invention. The antibody or binding portions thereof can be used alone or can be bound to a substance effective to kill cells that are in proximity to an elevated level of a chemokine of the present invention or that bound to the chemokine. In this method, these antibodies or binding portions thereof are contacted

with the cells under conditions effective to permit killing or ablating of the cells. In its preferred form, such contacting is carried out in a living patient by administering (e.g., orally or parenterally) the antibody or binding portion thereof to the patient under conditions effective to permit localization of the antibody or binding portion thereof to
5 tissues having elevated concentrations of the subject chemokine and killing or ablating of cells within such tissues.

Antibodies and binding portions thereof suitable for either killing, ablating, or detecting diseased breast tissue cells include antibodies, such as monoclonal or polyclonal antibodies. In addition, antibody fragments, half-antibodies, hybrid
10 derivatives, and other molecular constructs may be utilized. These antibodies and binding portions recognize and bind to chemokines of the present invention, which are associated with breast disease.

Monoclonal antibody production may be effected by techniques which are well-known in the art. Basically, the process involves first obtaining immune cells
15 (lymphocytes) from the spleen of a mammal (e.g., mouse) which has been previously immunized with the antigen of interest either *in vivo* or *in vitro*. The antibody-secreting lymphocytes are then fused with (mouse) myeloma cells or transformed cells, which are capable of replicating indefinitely in cell culture, thereby producing an immortal, immunoglobulin-secreting cell line. The resulting fused cells, or hybridomas, are
20 cultured, and the resulting colonies screened for the production of the desired monoclonal antibodies. Colonies producing such antibodies are cloned and grown either *in vivo* or *in vitro* to produce large quantities of antibody. A description of the theoretical basis and practical methodology of fusing such cells is set forth in Kohler and Milstein, Nature 256:495 (1975), which is hereby incorporated by reference.

25 Mammalian lymphocytes are immunized by *in vivo* immunization of the animal (e.g., a mouse) with the protein or polypeptide of the present invention. Such immunizations are repeated as necessary at intervals of up to several weeks to obtain a sufficient titer of antibodies. Following the last antigen boost, the animals are sacrificed and spleen cells removed.

30 Fusion with mammalian myeloma cells or other fusion partners capable of replicating indefinitely in cell culture is effected by standard and well-known techniques, for example, by using polyethylene glycol ("PEG") or other fusing agents (see Milstein and Kohler, Eur. J. Immunol. 6:511 (1976), which is hereby incorporated by reference).

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This immortal cell line, which is preferably murine, but may also be derived from cells of other mammalian species, including but not limited to rats and humans, is selected to be deficient in enzymes necessary for the utilization of certain nutrients, to be capable of rapid growth, and to have good fusion capability. Many such cell lines are known to those skilled in the art, and others are regularly described.

Procedures for raising polyclonal antibodies are also well known. Typically, such antibodies can be raised by administering the protein or polypeptide of the present invention subcutaneously to New Zealand white rabbits which have first been bled to obtain pre-immune serum. The antigens can be injected at a total volume of 100 μ l per site at six different sites. Each injected material will contain adjuvants with or without pulverized acrylamide gel containing the protein or polypeptide after SDS-polyacrylamide gel electrophoresis. The rabbits are then bled two weeks after the first injection and periodically boosted with the same antigen three times every six weeks. A sample of serum is then collected 10 days after each boost. Polyclonal antibodies are then recovered from the serum by affinity chromatography using the corresponding antigen to capture the antibody. This and other procedures for raising polyclonal antibodies are disclosed in E. Harlow, et. al., editors, Antibodies: A Laboratory Manual (1988), which is hereby incorporated by reference.

In addition to utilizing whole antibodies, the processes of the present invention encompass use of binding portions of such antibodies. Such binding portions include Fab fragments, $F(ab')_2$ fragments, and Fv fragments. These antibody fragments can be made by conventional procedures, such as proteolytic fragmentation procedures, as described in Goding, Monoclonal Antibodies: Principles and Practice, pp. 98-118, New York:Academic Press (1983), which is hereby incorporated by reference.

It is particularly preferred to use antibodies which recognize a chemokine having an amino acid sequence corresponding to SEQ. ID. No. 1 or a peptide having an amino acid sequence corresponding to SEQ. ID. No. 3, SEQ. ID. No. 4, or SEQ. ID. No. 5. These antibodies can be used alone or as a component in a mixture with other antibodies or other biological agents to treat or image tissues containing a mammary associated chemokine of the present invention.

Regardless of whether the antibodies or binding portions thereof are used for treatment or *in vivo* detection, they can be administered orally, parenterally, subcutaneously, intravenously, intramuscularly, intraperitoneally, by intranasal

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instillation, by intracavitary or intravesical instillation, intraocularly, intraarterially, intralesionally, or by application to mucous membranes, such as, that of the nose, throat, and bronchial tubes. They may be administered alone or with pharmaceutically or physiologically acceptable carriers, excipients, or stabilizers, and can be in solid or liquid
5 form such as, tablets, capsules, powders, solutions, suspensions, or emulsions.

The solid unit dosage forms can be of the conventional type. The solid form can be a capsule, such as an ordinary gelatin type containing the antibodies or binding portions thereof of the present invention and a carrier, for example, lubricants and inert fillers such as, lactose, sucrose, or cornstarch. In another embodiment, these
10 compounds are tableted with conventional tablet bases such as lactose, sucrose, or cornstarch in combination with binders like acacia, cornstarch, or gelatin, disintegrating agents, such as cornstarch, potato starch, or alginic acid, and a lubricant, like stearic acid or magnesium stearate.

The antibody or binding portion thereof of the present invention may also
15 be administered in injectable dosages by solution or suspension of these materials in a physiologically acceptable diluent with a pharmaceutical carrier. Such carriers include sterile liquids, such as water and oils, with or without the addition of a surfactant and other pharmaceutically and physiologically acceptable carrier, including adjuvants, excipients or stabilizers. Illustrative oils are those of petroleum, animal, vegetable, or
20 synthetic origin, for example, peanut oil, soybean oil, or mineral oil. In general, water, saline, aqueous dextrose and related sugar solution, and glycols, such as propylene glycol or polyethylene glycol, are preferred liquid carriers, particularly for injectable solutions.

For use as aerosols, the antibody or binding portion thereof of the present invention in solution or suspension may be packaged in a pressurized aerosol container
25 together with suitable propellants, for example, hydrocarbon propellants like propane, butane, or isobutane with conventional adjuvants. The materials of the present invention also may be administered in a non-pressurized form such as in a nebulizer or atomizer.

As indicated above, the antibody or binding portion thereof may be used to detect, *in vivo*, breast disease in a patient. This is preferably achieved by labeling the
30 antibody or binding portion thereof, administering the labeled antibody or binding portion thereof to the patient, and then imaging the patient.

Examples of labels useful for diagnostic imaging in accordance with the present invention are radiolabels such as ^{131}I , ^{111}In , ^{123}I , $^{99\text{m}}\text{Tc}$, ^{32}P , ^{125}I , ^3H , ^{14}C , and

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¹⁸⁸Rh. fluorescent labels such as fluorescein and rhodamine, nuclear magnetic resonance active labels, positron emitting isotopes detectable by a positron emission tomography ("PET") scanner, chemiluminescers such as luciferin, and enzymatic markers such as peroxidase or phosphatase. Short-range radiation emitters, such as isotopes detectable by short-range detector probes can also be employed. The antibody or binding portion thereof can be labeled with such reagents using techniques known in the art. For example, see Wensel and Meares, Radioimmunoimaging and Radioimmunotherapy, New York:Elsevier (1983), which is hereby incorporated by reference, for techniques relating to the radiolabeling of antibodies. See also, Colcher et al., "Use of Monoclonal Antibodies as Radiopharmaceuticals for the Localization of Human Carcinoma Xenografts in Athymic Mice", Meth. Enzymol. 121:802-816 (1986), which is hereby incorporated by reference.

Detecting the presence of a complex between an antibody or binding portion thereof and a chemokine of the present invention can be carried out by any conventional method for detecting antigen-antibody reactions, examples of which can be found, e.g., in Klein, Immunology, New York:John Wiley & Sons, pp. 394-407 (1982), which is hereby incorporated by reference. For *in vitro* detection of breast disease, the formation of a complex between the antibody and chemokine present in the tissue of fluid sample can be detected by enzyme linked assays, such as ELISA assays. Briefly, the antibody/chemokine complex is contacted with a second antibody which recognizes a portion of the antibody that is complexed with the chemokine. Generally, the second antibody is labeled so that its presence (and, thus, the presence of an antibody/chemokine complex) can be detected. Alternatively, the antibody or binding portion thereof can be bound to a label effective to permit detection of the chemokine upon binding of the antibody or binding portion thereof to the chemokine. Suitable labels include, fluorophores, chromophores, radiolabels, and the like.

For example, a radiolabeled antibody or binding portion thereof of this invention can be used for *in vitro* diagnostic tests. The specific activity of a tagged antibody or binding portion thereof depends upon the half-life and isotopic purity of the radioactive label and how the label is incorporated into the antibody or its binding portion. Table I lists several commonly-used isotopes, their specific activities and half-lives. In immunoassay tests, the higher the specific activity, in general, the better the sensitivity.

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TABLE 1

5	Isotope	Specific Activity of Pure Isotope (Curies/mole)	Half-Life
	^{14}C	6.25×10^1	5720 years
	^3H	2.01×10^4	12.5 years
	^{35}S	1.50×10^6	87 days
10	^{125}I	2.18×10^6	60 days
	^{32}P	3.16×10^6	14.3 days
	^{131}I	1.62×10^7	8.1 days

Procedures for labeling antibodies and binding portions thereof with the radioactive isotopes listed in Table 1 are generally known in the art. Tritium labeling procedures are described in U.S. Patent No. 4,302,438 to Zech, which is hereby incorporated by reference. Iodinating, tritium labeling, and ^{35}S labeling procedures especially adapted for murine monoclonal antibodies are described in Goding, Monoclonal Antibodies: Principles and Practice, pp. 124-126, New York:Academic Press (1983) and the references cited therein, which are hereby incorporated by reference.

Other procedures for iodinating antibodies or binding portions thereof are described in Hunter et al., Nature 144:945 (1962), David et al., Biochemistry 13:1014-1021 (1974), U.S. Patent No. 3,867,517 to Ling, and U.S. Patent No. 4,376,110 to David et al., which are hereby incorporated by reference. Radiolabeling elements which are useful in imaging include ^{123}I , ^{131}I , ^{111}In , and $^{99\text{m}}\text{Tc}$, for example. Procedures for iodinating antibodies or binding portions thereof are described in Greenwood et al., Biochem. J. 89:114-123 (1963); Marchalonis, Biochem. J. 113:299-305 (1969); and Morrison et al., Immunochemistry 289-297 (1971), which are hereby incorporated by reference.

Procedures for $^{99\text{m}}\text{Tc}$ -labeling are described by Rhodes et al. in Burchiel et al., eds., Tumor Imaging: The Radioimmunochemical Detection of Cancer, New York:Masson 111-123 (1982) and the references cited therein, which are hereby incorporated by reference. Procedures suitable for ^{111}In -labeling antibodies or binding portions thereof are described by Hnatowich et al., J. Immunol. Methods 65:147-157 (1983), Hnatowich et al., J. Applied Radiation 35:554-557 (1984), and Buckley et al., F.E.B.S. 166:202-204 (1984), which are hereby incorporated by reference.

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The antibodies or binding portions thereof of the present invention can be used and sold together with equipment to detect the particular label as a kit for *in vitro* detection of breast disease.

In the case of a radiolabeled antibody or binding portion thereof, the
5 antibody or binding portion thereof is administered to the patient, is localized to the region of the patient where diseased breast cells produce increased levels of the subject chemokines, and is detected or "imaged" *in vivo* using known techniques such as radionuclear scanning using e.g., a gamma camera or emission tomography. See e.g., Bradwell et al., "Developments in Antibody Imaging" in Baldwin et al., eds., Monoclonal
10 Antibodies for Cancer Detection and Therapy, pp. 65-85, New York:Academic Press (1985), which is hereby incorporated by reference. Alternatively, a positron emission transaxial tomography scanner, such as the one designated Pet VI located at Brookhaven National Laboratory, can be used where the radiolabel emits positrons (e.g., ^{11}C , ^{18}F , ^{15}O , and ^{13}N).

15 Fluorophore and chromophore labeled antibodies and binding portions thereof can be prepared from standard moieties known in the art. Since antibodies and other proteins absorb light having wavelengths up to about 310 nm, the fluorescent moieties should be selected to have substantial absorption at wavelengths above 310 nm and preferably above 400 nm. A variety of suitable fluorescers and chromophores are
20 described in Stryer, Science, 162:526 (1968) and Brand et al., Annual Review of Biochemistry, 41:843-868 (1972), which are hereby incorporated by reference. The antibodies and binding portions thereof can be labeled with fluorescent chromophore groups by conventional procedures such as those disclosed in U.S. Patent Nos. 3,940,475 to Gross, 4,289,747 to Chu, and 4,376,110 to David et al., which are hereby incorporated
25 by reference.

One group of fluorescers having a number of the desirable properties described above are the xanthene dyes, which include the fluoresceins derived from 3,6-dihydroxy-9-hexylxanthhydrol and resamines and rhodamines derived from 3,6-diamino-9-phenylxanthhydrol and lissanime rhodamine B. The rhodamine and
30 fluorescein derivatives of 9-o-carboxyphenylxanthhydrol have a 9-o-carboxyphenyl group. Fluorescein compounds having reactive coupling groups such as amino and isothiocyanate groups such as fluorescein isothiocyanate and fluorescamine are readily

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available. Another group of fluorescent compounds are the naphthylamines, having an amino group in the α or β position.

Antibodies and binding portions thereof can be labeled with fluorochromes or chromophores by the procedures described in Goding, Monoclonal Antibodies: Principles and Practice, pp. 208-249, New York:Academic Press (1983), which is hereby incorporated by reference. The antibodies and binding portions thereof can be labeled with an indicating group containing the NMR-active ^{19}F atom, or a plurality of such atoms inasmuch as (i) substantially all of naturally abundant fluorine atoms are the ^{19}F isotope and, thus, substantially all fluorine-containing compounds are NMR-active; (ii) many chemically active polyfluorinated compounds such as trifluoroacetic anhydride are commercially available at relatively low cost, and (iii) many fluorinated compounds have been found medically acceptable for use in humans such as the perfluorinated polyethers utilized to carry oxygen as hemoglobin replacements. After permitting such time for incubation, a whole body NMR determination is carried out using an apparatus such as one of those described in Pykett, Scientific American, 246:78-88 (1982), which is hereby incorporated by reference, to locate and image regions of elevated chemokine concentration.

The antibodies and binding portions thereof can also be utilized to treat breast disease *in vivo*. This involves administering to a patient in need of such treatment the antibodies or binding portions thereof by themselves or with a cytotoxic drug to which the antibodies and binding portions thereof are bound. Since the antibodies and binding portions thereof recognize the subject chemokines, diseased breast cells, which are in proximity to elevated levels of the subject chemokines which they produce, are destroyed. Caution must be exercised, however, as such administration may destroy normal cells which are in proximity to the chemokines produced by the diseased breast cells.

The antibodies and binding portions thereof of the present invention may be used to deliver a variety of cytotoxic drugs including therapeutic drugs, a compound emitting radiation, molecules of plants, fungal, or bacterial origin, biological proteins, and mixtures thereof.

Enzymatically active toxins and fragments thereof are exemplified by diphtheria toxin A fragment, nonbinding active fragments of diphtheria toxin, exotoxin A (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, α -sacrin, certain *Aleurites fordii* proteins, certain Dianthin proteins, *Phytolacca*

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americana proteins (PAP, PAPII and PAP-S), *Morodica charantia* inhibitor, curcin, crotin, *Saponaria officinalis* inhibitor, gelonin, mitogillin, restrictocin, phenomycin, and enomycin, for example. Procedures for preparing enzymatically active polypeptides of the immunotoxins are described in W084/03508 and W085/03508, which are hereby
5 incorporated by reference. Certain cytotoxic moieties are derived from adriamycin, chlorambucil, daunomycin, methotrexate, neocarzinostatin, and platinum, for example.

Procedures for conjugating the antibodies and binding portions thereof with the cytotoxic agents have been previously described. Procedures for conjugating chlorambucil with antibodies are described in Flechner, European Journal of Cancer
10 9:741-745 (1973); Ghose et al., British Medical Journal 3:495-499 (1972); and Szekerke et al., Neoplasma 19:211-215 (1972), which are hereby incorporated by reference. Procedures for conjugating daunomycin and adriamycin to antibodies are described in Hurwitz et al., Cancer Research 35:1175-1181 (1975) and Arnon et al. Cancer Surveys
15 1:429-449 (1982), which are hereby incorporated by reference. Procedures for preparing antibody-ricin conjugates are described in U.S. Patent No. 4,414,148 to Jansen et al. and in Osawa et al. Cancer Surveys 1:373-388 (1982) and the references cited therein, which are hereby incorporated by reference. Coupling procedures are also described in EP
86309516.2, which is hereby incorporated by reference.

The use of the subject antibodies and binding portions thereof can also be
20 used in a drug/prodrug treatment regimen. In this method, for example, a first antibody or binding portion thereof according to the present invention is conjugated with a prodrug which is activated only when in close proximity with a prodrug activator. The prodrug activator is conjugated with a second antibody or binding portion thereof, preferably one which binds to diseased breast cells or to other biological materials associated with
25 diseased breast cells (e.g., another protein produced by diseased breast cells). Drug-prodrug pairs suitable for use in the practice of the present invention are described in Blakely et al., "ZD2767, an Improved System for Antibody-directed Enzyme Prodrug Therapy That Results in Tumor Regressions in Colorectal Tumor Xenografts," Cancer Research 56:3287-3292 (1996), which is hereby incorporated by reference.

30 Alternatively, the antibody or binding portion thereof can be coupled to high energy radiation emitters, for example, a radioisotope, such as ¹³¹I, a γ -emitter, which, when localized at the diseased breast tissue site, results in a killing of several cell diameters. See, e.g., Order, "Analysis, Results, and Future Prospective of the Therapeutic

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Use of Radiolabeled Antibody in Cancer Therapy" in Baldwin et al., eds., Monoclonal Antibodies for Cancer Detection and Therapy, pp 303-316, New York:Academic Press (1985), which is hereby incorporated by reference. Other suitable radioisotopes include α -emitters, such as ^{212}Bi , ^{213}Bi , and ^{211}At , and β -emitters, such as ^{186}Re and ^{90}Y .

5 Where the antibodies or binding portions thereof are used alone to treat breast disease, such treatment can be effected by initiating endogenous host immune functions, such as complement-mediated or antibody-dependent cellular cytotoxicity.

 The antibodies or binding portions thereof of the present invention can be used in conjunction with other therapeutic treatment modalities. Such other treatments
10 include surgery, radiation, cryosurgery, thermotherapy, hormone treatment, chemotherapy, vaccines, and other immunotherapies.

 Also encompassed by the present invention is a method of treating breast disease which involves using the antibodies and binding portions thereof without cytotoxic agents for prophylaxis. For example, the antibodies and binding portions
15 thereof can be used to prevent or delay development or progression of breast disease by binding to the chemokines of the present invention and, thus, inhibiting their biological activity.

 Another aspect of the present invention relates to an isolated nucleic acid molecule which encodes a chemokine of the present invention. The encoded chemokine
20 is preferably one that is preferentially expressed in breast tissue or one which can be detected in breast milk. The encoded chemokine can include from about 100 to about 132 amino acids, preferably from about 105 to about 127 amino acids, more preferably, about 105 or 127 amino acids; can have a deduced molecular weight of from about 10 to about 16 kDa, preferably from about 12 kDa to about 14 kDa; and can have a deduced isoionic
25 point of from about pH 10.1 to about pH 10.7, preferably about 10.4. The term "isolated nucleic acid molecules" is intended to refer to nucleic acid molecules that are substantially free of the biological materials with which they are naturally found. The term "nucleic acid" is meant to refer to polydeoxyribonucleotides ("DNA"), which contain 2-deoxy-D-ribose, to polyribonucleotides ("RNA"), which contain D-ribose, and
30 to any other type of polynucleotide which is an N-glycoside of a purine or pyrimidine base or a modified purine or pyrimidine base. The term "nucleic acid" refers only to the primary structure of the molecule, and, thus, it is meant to include double- and single-stranded DNA as well as double- and single-stranded RNA. There is no intended

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distinction in length between the terms "nucleic acid" and "oligonucleotide". and these terms are used interchangeably herein.

The nucleic acid molecule can be a DNA or RNA molecule which encodes a chemokine having an amino acid sequence corresponding to SEQ. ID. No. 1. One such
5 nucleic acid molecule has a nucleotide sequence corresponding to SEQ. ID. No. 6 as follows:

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AACATCCTCA CTTGTGTTGC TGTCAGTGCC TGTANGGCAG GCAGGAATGC
AGCAGAGAGG ACTCGCCATC GTGGCCTTGG CTGTCTGTGC GGCCCTACAT
10 GCCTCAGAAG CCATACTTCC CATTGCCTCC AGCTGTTGCA CGGAGGTTTC
ACATCATATT TCCAGAAGGC TCCTGGAAAG AGTGAATATG TGTGCGCATCC
AGAGAGCTGA TGGGGATTGT GACTTGGCTG CTGTCATCCT TCATGTCAAG
CGCNGAAGAA TCTGTGTCAG CCCGCACAAC CATACTGTTA AGCAGTGGAT
GAAAGTGCAA GCTGCCAANA AAAATGGTAA AGGAAATGTT TGCCACAGGA
15 AGAAACACCA TGGCAAGAGG AACAGTAACA GGGCACATCA GGGGAAACAC
GAAACATACG GCCATAAAAC TCCTTATTAG AGAATCTACA GATAAATCTA
CAGAGACAAT CCCCCAAGTG GACTTGGCCA TGATTGGTTG TAAGTTTATC
ATCTGAATTC TCCTTATTGT AGACAACAGA ACAAACAAA ATATTGGTTT
TTAAAAAATG AACAAATTGT CCGTATGCAA ATGTACCCAA TAATATACTC
20 CACTGGAAAA TGAAATGAAA AAANNATACT GGCTGGGTAT GGTGGGTCCC
CCCTTTTATC CCANNNNCTT CGGGAGGCAG AGGCAGGAGG ATCACTTGAG
ACCAGGANTT NGAGACNAGC TNGGGGCAAA ANAGCAANGA CNTCATTTNT
ACAAACNAAA AAAAANNTTG GCCCGGCNTG GTAGNACTTG CNTATAATCC
CAGCNACATG GGAGGTNGAG GTGGGAGGAT CACTTGAGTC TGGGNGAGTT
25 NGAGGTNGCA GTGAGCAGCN TGGGTGACAG AATGNAGACC NTGTCTCTAA
AAATAATAAT AATAATGATA GTGTATATCT TCATATAATA TTTTAAGNAG
GAGCATATAG ATATAACTTN CTCCCAACTT TTTAATTATA GTTTTCCAAA
CTTACAGAGA AGTTAAAAGA ATGGTACAAT GAACATCTAT ATATCTTTCA
CCACAATATT AATCATTGTT AATATTGTGC CACATTTGCT TTCTCTCTCC
30 TCTCTTG GTA GGGGTNCAA TATAAAATAT TATAACTTTT AAAATATATC
TTGTTTTGCT AACCATTGGA AAATAAGTTG CAAAATCAT GACACTTCAC
CCCTAGTTTC TTTTNGGTGT TATAACTTGA CATACCCTAA AATAAGACA
TTTTTCTACA TAATCACCTT ATCAGTTTTA TACCTAAAAA ATTAATAATT
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TCATCTAATA TATTCCATAT TCAAATTTTC CCAACTATTT AGAGAGCATT
TTATGTAGTT TTTTTCAC TCCAGTAATC AATCAAGGTN GACATACATA
TTGCAAATAA TTGTTATTTT TCTTTAATAT CTTTCAATCT AAGAAAGTTC
CTCTGTCTTT TTTTTTTAAT TTTTAAATTT ATTTTGTTGA GGGAGGGTCT
5 TGCTGTGTCT TCCAGGCTGG AGTGCAGTGG CACAATTTTG ATTTTGGCTC
ACTGAAGCCT CAACTTTAGG GCTCAAGCAA TCCTCCCACC TCAGCCTNCC
CGAGTATCTG GGATCAAGGT GCATACCCAC CACACCTGGC TAATTTTGTT
TATTTTTTGT AGAGACAGGG TCTCACTATG TTGCCCAGGT TGATCTCAAA
CTCCTGGGCT CAAGCGATCC TCCCACCTTA GCCTCCCAA GTACTGGGAT
10 TATAGGTGTG AGCCACAGTG CCTGGCCTAA TTATTTTCTT GTGATCAAAT
TCAGGTTTAA TGTTTTTGGT TAAGAATTTT CTACGTGAAT TCGTGTACTT
ATTTTGTCT TTAGAGTTCA TAAATATTAG GGTTTATTTT CTAAATAGAA
TAGTTTAAAC TAAATATAAC TTCAAAACGT CTAGTTTGAG TAGCTACCGT
TGTTTTGATT GAAATTTTCT GATACTGAAA AGAACAAAAA GCCTGCCTTT
15 CTGCCCCANAA CSNNTTGCTT CCCCCAGTNA GTTCTTGGNG CAGNACTAGT
TAGGGNCCCA GAGTTNGGCC TTNGKGTGG TGATTTTANG YTCTGCCTAA
ACAAGGNGCN WACATYTTTT AGCTCCTATT CCACCYTTCT NAMAMGTTTT
TGTTGKGTG TGNTTGTTTT TTTKGAGACA GRRNTNAYT CTGTTTGCCC
ARGCTGGART TGCAGTGGCA CAATYTNGGY TNCATTGCAA CYTCNGCYTC
20 CSSGCCGTTT AAKTGATYYT CTTGCTCAG CYTCCCCAAG TAANTGATAT
TACAGGNGCC CAGCCACCAM ACCCCGNTGA WTTTGTATT TTTARTARAR
AMRGGGTTTT CCCGNTTGG CNGGGCTGGT CTCNAANTCC TTGAMCTCNA
KTGAACCACC CGCCTGTGCC YCCCAAANTG CTGGAATTAC CANCGTTGAN
CCACCATGCC GGGCYCACAC GTTTGARTTT GANACCATTG TNCCATTCTT
25 CTTTTGGCCT YTTTTTTNTC CATAGNNGCT TCAAGATAGA TANGTAAGRG
CCCAGTAGTN GTTCWTARGA AGCNMATAGR RANCRGGARC CANTTTNATC
AGGTGGGCAG GTGTCCNNGG CYTCCCTGCT GGYTNNTCCC AAGCGGTGGT
GTTGCCARGA NKTNTTGGAR GTGATAATGG GANANACCAG NAGGCMCTGA
GTYN CNNTAG GTTNAAATGC CACCAAAACT GGCCTTTGGC CTAATATCCY
30 YCNTTGAMTA NTTARCATT AWTTTATTWA TTTNCCTGAC ATTTNTGCM
NCCTTTGTWT TTNTATTTCC NCTNTATARA WGARGAAATT TGAGGNTYTT
ARAGGTAAAA TGANTGCNC NRGTTNACMC AGGAAGTGGC NRARANAANC
TTTTTANATN MGAAAAAATT AATAAAATAT AATATGAGAG TAACTTAAAA

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TATTAATAAA CCACAATTTT AAATTAATTA ACCGTGATAA CCAACATTAA
TAAAAGTTAA GATACCAAAA CACTGGTGTN TAATTTTTTN AACTAACAAN
TTGAATTATT TTCCATTTTA AATTAATTAA CCGTGATAAC CAACATTAAT
AAAAGTTAAG ATACCGN

5

Another such nucleic acid molecule has a nucleotide sequence
corresponding to SEQ. ID. No. 7 as follows:

TGCAGCAGAG AGGACTCGCC ATCGTGGCCT TGGCTGTCTG TCGGGCCCTA
10 CATGCCTCAG AAGCCATACT TCCATTGCC TCCAGCTGTT GCACGGAGGT
TTCACATCAT ATTTCCAGAA GGCTCCTGGA AAGAGTGAAT ATGTGTCGCA
TCCAGAGAGC TGATGGGGAT TGTGACTTGG CTGCTGTCAT CCTTCATGTC
AAGCGCNGAA GAATCTGTGT CAGCCCGCAC AACCATACTG TTAAGCAGTG
GATGAAAGTG CAAGCTGCCA ANAAAAATGG TAAAGGAAAT GTTTGCCACA
15 GGAAGAAACA CCATGGCAAG AGGAACAGTA ACAGGGCACA TCAGGGGAAA
CACGAAACAT ACGGCCATAA AACTCCTTAT T

This nucleic acid represents an open reading frame of the nucleic acid molecule having a
nucleotide sequence corresponding to SEQ. ID. No. 6.

20

The above isolated nucleic acid molecules of the present invention which
encode for chemokines of the present invention can be used along with conventional
recombinant methods to produce isolated chemokines of the present invention

Briefly, this is carried out by incorporating any one of the DNA molecules
encoding chemokines of the present invention in cells using conventional recombinant
25 DNA technology. This involves inserting the selected DNA molecule into an expression
system to which that DNA molecule is heterologous (i.e., not normally present). The
heterologous DNA molecule is inserted into the expression system or vector in proper
orientation and correct reading frame. The vector contains the necessary elements for the
transcription and translation of the inserted protein-coding sequences.

30

U.S. Patent No. 4,237,224 to Cohen and Boyer, which is hereby
incorporated by reference, describes the production of expression systems in the form of
recombinant plasmids using restriction enzyme cleavage and ligation with DNA ligase.
These recombinant plasmids are then introduced by means of transformation and

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replicated in unicellular cultures including procaryotic organisms and eucaryotic cells grown in tissue culture.

Recombinant genes may also be introduced into viruses, such as vaccinia virus. Recombinant viruses can be generated by transfection of plasmids into cells infected with virus.

Suitable vectors include, but are not limited to, the following viral vectors such as lambda vector system gt11, gt WES.tB, Charon 4, and plasmid vectors such as pBR322, pBR325, pACYC177, pACYC184, pUC8, pUC9, pUC18, pUC19, pLG339, pR290, pKC37, pKC101, SV 40, pBluescript II SK +/- or KS +/- (see "Stratagene Cloning Systems" Catalog (1993) from Stratagene, La Jolla, Calif, which is hereby incorporated by reference), pQE, pIH821, pGEX, pET series (see Studier et. al., "Use of T7 RNA Polymerase to Direct Expression of Cloned Genes" in Gene Expression Technology, vol. 185 (1990), which is hereby incorporated by reference) and any derivatives thereof. Recombinant molecules can be introduced into cells via transformation, particularly transduction, conjugation, mobilization, or electroporation. The DNA sequences are cloned into the vector using standard cloning procedures in the art, as described by Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Springs Harbor, New York: Cold Springs Laboratory Press (1982), which is hereby incorporated by reference.

A variety of host-vector systems may be utilized to express the protein-encoding sequence(s). Primarily, the vector system must be compatible with the host cell used. Host-vector systems include but are not limited to the following: bacteria transformed with bacteriophage DNA, plasmid DNA, or cosmid DNA; microorganisms such as yeast containing yeast vectors; mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus). The expression elements of these vectors vary in their strength and specificities. Depending upon the host-vector system utilized, any one of a number of suitable transcription and translation elements can be used.

Different genetic signals and processing events control many levels of gene expression (e.g., DNA transcription and messenger RNA (mRNA) translation).

Transcription of DNA is dependent upon the presence of a promoter which is a DNA sequence that directs the binding of RNA polymerase and thereby promotes mRNA synthesis. The DNA sequences of eucaryotic promoters differ from those of

procaryotic promoters. Furthermore, eucaryotic promoters and accompanying genetic signals may not be recognized in or may not function in a procaryotic system, and, further, procaryotic promoters are not recognized and do not function in eucaryotic cells.

Similarly, translation of mRNA in procaryotes depends upon the presence
5 of the proper procaryotic signals which differ from those of eucaryotes. Efficient translation of mRNA in procaryotes requires a ribosome binding site called the Shine-Dalgarno ("SD") sequence on the mRNA. This sequence is a short nucleotide sequence of mRNA that is located before the start codon, usually AUG, which encodes the amino-terminal methionine of the protein. The SD sequences are complementary to the 3'-end
10 of the 16S rRNA (ribosomal RNA) and probably promote binding of mRNA to ribosomes by duplexing with the rRNA to allow correct positioning of the ribosome. For a review on maximizing gene expression, see Roberts et al., Methods in Enzymology 68:473 (1979), which is hereby incorporated by reference.

Promoters vary in their "strength" (i.e., their ability to promote
15 transcription). For the purposes of expressing a cloned gene, it is desirable to use strong promoters in order to obtain a high level of transcription and, hence, expression of the gene. Depending upon the host cell system utilized, any one of a number of suitable promoters may be used. For instance, when cloning in *E. coli*, its bacteriophages, or plasmids, promoters such as the T7 phage promoter, *lac* promoter, *trp* promoter, *recA*
20 promoter, ribosomal RNA promoter, the P_R and P_L promoters of coliphage lambda and others, including but not limited, to *lacUV5*, *ompF*, *bla*, *lpp*, and the like, may be used to direct high levels of transcription of adjacent DNA segments. Additionally, a hybrid *trp-lacUV5* (*tac*) promoter or other *E. coli* promoters produced by recombinant DNA or other synthetic DNA techniques may be used to provide for transcription of the inserted gene.

25 Bacterial host cell strains and expression vectors may be chosen which inhibit the action of the promoter unless specifically induced. In certain operons, the addition of specific inducers is necessary for efficient transcription of the inserted DNA. For example, the *lac* operon is induced by the addition of lactose or IPTG (isopropylthio-beta-D-galactoside). A variety of other operons, such as *trp*, *pro*, etc., are under different
30 controls.

Specific initiation signals are also required for efficient gene transcription and translation in procaryotic cells. These transcription and translation initiation signals may vary in "strength" as measured by the quantity of gene specific messenger RNA and

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protein synthesized, respectively. The DNA expression vector, which contains a promoter, may also contain any combination of various "strong" transcription and/or translation initiation signals. For instance, efficient translation in *E. coli* requires a Shine-Dalgarno ("SD") sequence about 7-9 bases 5' to the initiation codon (ATG) to provide a ribosome binding site. Thus, any SD-ATG combination that can be utilized by host cell ribosomes may be employed. Additionally, any SD-ATG combination produced by recombinant DNA or other techniques involving incorporation of synthetic nucleotides may be used.

Once the desired isolated DNA molecule encoding a chemokine according to the present invention has been cloned into an expression system, it is ready to be incorporated into a host cell. Such incorporation can be carried out by the various forms of transformation noted above, depending upon the vector/host cell system. Suitable host cells include, but are not limited to, bacteria, virus, yeast, mammalian cells, and the like.

Recombinant DNA technology can also be used to produce fragments of the above chemokines, such as the above-referenced peptides. For example, subclones of the gene encoding a subject chemokine are produced by conventional molecular genetic manipulation by subcloning gene fragments. The subclones then are expressed *in vitro* or *in vivo* in bacterial cells to yield a smaller peptide that can be tested for its antigenic activity (i.e., capacity to be used as an antigen to raise antibodies which recognize an antigenic portion of the chemokine).

As an alternative, protein fragments can be produced by digestion of a full-length subject chemokine with proteolytic enzymes like chymotrypsin, *Staphylococcus* proteinase A, or trypsin. Different proteolytic enzymes are likely to cleave proteins at different sites based on the amino acid sequence of the protein. Some of the fragments that result from proteolysis may have antigenic activity.

In still another approach, based on knowledge of the primary structure of the subject chemokines, fragments of the encoding gene may be synthesized by using the polymerase chain reaction ("PCR") technique together with specific sets of primers chosen to represent particular portions of the protein. These then would be cloned into an appropriate vector to facilitate expression of a peptide having, for example, antigenic activity.

Chemical synthesis can also be used to make suitable fragments. Such a synthesis is carried out using known amino acid sequences for the chemokines of the

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present invention. Alternatively, subjecting a full length subject chemokine to high temperatures and pressures will produce fragments. These fragments can then be separated by conventional procedures (e.g., chromatography and SDS-PAGE).

The chemokines of the present invention and their fragments can optionally be modified by, for example, the deletion or addition of amino acids that have minimal influence on the properties, secondary structure, and hydrophobic nature of the chemokine or fragments. For example, a chemokine or peptide of the present invention can be conjugated to a signal (or leader) sequence at the N-terminal end of the chemokine which co-translationally or post-translationally directs transfer of the protein. The chemokine or peptide can also be conjugated to a linker or other sequence for ease of protein synthesis, purification, or identification. The peptides of the present invention can also include, in addition to the antigenic portion of the chemokine, other amino acid sequences, such as T-cell antigenic stimuli and other amino acid sequences which increase the peptide's immunogenicity.

As indicated above, the chemokines and peptides of the present invention are preferably produced in purified form (preferably at least about 80%, more preferably 90% pure) by conventional techniques. The chemokines or peptides of the present invention are preferably produced in purified form by conventional techniques, of which the following is one example. To isolate the proteins, an *E. coli* host cell carrying a recombinant plasmid is propagated and homogenized, and the homogenate is centrifuged to remove bacterial debris. The supernatant is then subjected to sequential ammonium sulfate precipitation. The fraction containing the chemokines or peptides of the present invention is subjected to gel filtration in an appropriately sized dextran or polyacrylamide column to separate the chemokines or peptides. If necessary, the chemokine or peptide fraction may be further purified by ion exchange chromatography and/or HPLC.

As indicated above, the chemokines and peptides of the present invention can be used to raise antibodies which are useful in the detection and treatment of breast disease. Breast disease can also be treated using the peptides of the present invention by administering to a patient suffering from breast disease an effective amount of a peptide which binds to a cellular receptor for a chemokine of the present invention. Methods for identifying peptides which bind to cellular receptors of proteins having known amino acid sequences are well known to those skilled in the art and are described in, for example, Wells et al., "Selectivity and Antagonism of Chemokine Receptors," *J. Leukocyte Biol.*,

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59:53-60 (1996) and Horuk. "Molecular Properties of the Chemokine Receptor Family," Trends Pharmacol. Sci., 15:159-165 (1994), which are hereby incorporated by reference.

The present invention also relates to isolated nucleic acid molecules which, under stringent conditions, hybridize to a nucleic acid molecule encoding a chemokine of the present invention. Such isolated nucleic acid molecules include those which hybridize, under stringent hybridization conditions, to nucleic acid molecules (1) which encode chemokines that are preferentially expressed in breast tissue or that are detected in breast milk; (2) which encode chemokines which include from about 100 to about 132 amino acids, which have a deduced molecular weight of from about 10 to about 16 kDa, and which have a deduced isoionic point of from about pH 10.1 to about pH 10.7; (3) which encode chemokines which include from about 105 to about 127 amino acids, which have a deduced molecular weight of from about 12 to about 14 kDa, and which have an isoionic point of about pH 10.4; (4) which encode chemokines having an amino acid sequence corresponding to SEQ. ID. No. 1; (5) which have a nucleotide sequence corresponding to SEQ. ID. No. 6; and (6) which have a nucleotide sequence corresponding to SEQ. ID. No. 7. Preferably, the nucleic acid molecules which hybridize under stringent conditions to nucleic acid molecules encoding a chemokine of the present invention preferentially hybridize to nucleic acid molecules from breast tissue. That is, more of the chemokine of the present invention will hybridize, under stringent conditions, to nucleic acid molecules from breast tissue than to nucleic acid molecules from other tissues in the body.

The present invention also relates to isolated nucleic acid molecules which, under stringent conditions, hybridize to the complement of a nucleic acid molecule encoding a chemokine of the present invention.

"Stringent conditions", as used herein in relation to hybridization, mean approximately 35 °C to 70 °C, preferably about 50 °C, 55 °C, 60 °C, and/or 65 °C, in a salt solution of approximately 0.9 molar NaCl. These conditions are frequently represented by a wash stringency of 0.3 M NaCl, 0.03 M sodium citrate, 0.1% SDS at 70 °C to a DNA molecule encoding a chemokine of the present invention in a standard in situ hybridization assay. See Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor, New York: Cold Spring Harbor Laboratory (1989). In general, such sequences will be at least 95% homologous, often at least 98% homologous, and

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even at least 99% homologous with the sequences of DNA molecules encoding chemokines of the present invention.

Illustrative nucleic acid molecules include those which have a nucleotide sequence corresponding to ACACGAATTCACGTAGGAAATTCTTAACCAAAAACA
5 TTAAACCTGAATTTGATCACAAGAAAATAATTAGGCCAGGCACTGTGGCTCA
CACCTATAATCCCACT (SEQ. ID. No. 8), GAATTCACGTAGGAA ATTCTTAACC
(SEQ. ID. No. 9), ACTGGGATTATAGGTGTGAGCC (SEQ. ID. No. 10), and
GGAGAGAGCCGTATGTTTCGTGTTTCCCCTGATGTGCCCTGTTACTGTTCTCT
TGCCATGGTGTTCCTGTGGCAAACATTTCTTTACCATTTTTNTTGGCAG
10 CTTGCACTTTCATCCACTGCTTAACAGTATGGTTGTGCGGGCTGACACAGATT
NTTCTGCGCTTGACATGAAGGATGACAGCAGCCAAGTCACAATCCCCATCAG
CTCTCTGGATGCGACACATATTCACTCTTCCAGGAGCCTTCTGGAAATATGA
TGTGAAACCTCCGTGCAACAGCTGGAGGCAATGGGAAGTATGGCT (SEQ. ID.
No. 11), as well as to those which have a nucleotide sequence corresponding to a
15 complement of and of SEQ. ID. Nos. 8-11. Of course, as one skilled in the art will
recognize, although these exemplary nucleic acid molecules have a defined number of
nucleotides, one or more nucleotides may be added or deleted from a particular nucleic
acid molecule without great impact on its ability to hybridize with a nucleic acid molecule
encoding a chemokine of the present invention.

20 The exact size of nucleic acid molecules which hybridize under stringent
conditions to nucleic acid molecules encoding a chemokine of the present invention
depends on many factors and the ultimate use to which the nucleic acid molecule is to be
put. These nucleic acid molecules can be prepared by any suitable method, such as by
cloning and restriction of appropriate sequences and by direct chemical synthesis using,
25 for example, the phosphotriester method (described in, e.g., Narang et al., Meth.
Enzymol. 68:90-99 (1979), which is hereby incorporated by reference); the
phosphodiester method (described in, e.g., Brown et al., Meth. Enzymol. 68:109-151
(1979), which is hereby incorporated by reference); the diethylphosphoramidite method
(described in, e.g., Beaucage et al., Tetrahedron Lett. 22:1859-1862 (1981), which is
30 hereby incorporated by reference); and the solid support method (described in, e.g., U.S.
Patent No. 4,458,066 to Caruthers et al., which is hereby incorporated by reference).
These and other methods for synthesizing oligonucleotides are described in Goodchild,
Bioconjugate Chemistry 1(3):165-187 (1990), which is hereby incorporated by reference.

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The nucleic acid molecules which hybridize under stringent conditions to nucleic acid molecules encoding a chemokine of the present invention can be used as probes in hybridization assays to detect breast disease in a patient. For example, a sample of tissue or body fluid from the patient is contacted with a nucleic acid probe which, under stringent conditions, hybridizes to a nucleic acid molecule encoding a chemokine according to the present invention or to a complement thereof. The contacting is carried out under conditions effective to permit formation of a hybridization complex between the probe and breast tissue specific nucleic acid molecules (i.e., the nucleic acid molecules encoding chemokines of the present invention). Breast disease is then detected by detecting the hybridization complex.

As used herein, the term "probe" refers to an oligonucleotide which forms a duplex structure with a sequence of a target nucleic acid (e.g., a nucleic acid molecule which encodes a chemokine of the present invention) due to complementary base pairing. The probe will contain a hybridizing region, which is a region of the oligonucleotide corresponding to a region of the target sequence. A probe oligonucleotide either can consist entirely of the hybridizing region or can contain additional features which allow for the detection or immobilization of the probe but do not alter the hybridization characteristics of the hybridizing region. The term "probe" also refers to a set of oligonucleotides which provide sufficient sequence variants of the hybridization region to permit hybridization with each member of a given set of target sequence variants. Additionally, a probe can contain mismatches with some or all members of a given set of target sequence variants, provided that it contains sufficient regions of complementarity with each target sequence variant to permit hybridization with all target sequence variants under suitable conditions.

Samples of the patient's tissue or body fluids suitable for the use in the detection method using probes include those which are discussed above with regard to detection methods employing antibodies.

Detection of the hybridization complex can be carried out by a variety of conventional methods. These include electrophoresis, DNA sequencing, blotting, microplate hybridization, or microscopic visualization. Alternatively, the probe can have bound thereto a label, such as detectable functional nucleotide sequence (e.g., a T7 site, a restriction site, and the like) or one of the labels described above as suitable for use in the detection method of the present invention employing antibodies. Detection, in this case,

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involves detecting the presence of the label, for example using the techniques discussed above or by using one of the conventional methods for detecting detectable functional nucleotide sequences.

The nucleic acid molecules which hybridize under stringent conditions to
5 nucleic acid molecules encoding a chemokine of the present invention can also be used as primers in a DNA amplification assay to detect breast disease in a patient. For example, a sample of tissue or body fluid from the patient can be contacted with a nucleic acid primer which, under stringent conditions, hybridizes to a nucleic acid molecule encoding a chemokine according to the present invention or to a complement thereof. The sample of
10 tissue or body fluid from the patient in contact with the nucleic acid primer is then treated under conditions effective to amplify breast tissue specific nucleic acid molecules, and the breast tissue specific nucleic acid molecules, thus amplified, are then detected.

As used herein, the term "primer" refers to an oligonucleotide, whether natural or synthetic, capable of acting as a point of initiation of a DNA synthesis under
15 conditions which produce a primer extension product complementary to a nucleic acid strand is induced. Generally, the DNA synthesis is carried out in the presence of four different nucleoside triphosphates and an agent for polymerization (e.g., DNA polymerase or reverse transcriptase) in an appropriate buffer (e.g., Tris-HCl), and at suitable temperatures (e.g., at an annealing temperature of from about 45 to about 85 °C; at an extending temperature of from about 55 to about 75 °C; and at a melting temperature
20 of about 95 °C). The primer is preferably a single-stranded DNA. The optimal length of the primer depends on the primer's intended use but typically ranges from 15 to 35 nucleotides. Short primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with the template. A primer need not complement
25 the exact sequence of the template but must be sufficiently complementary to hybridize with a template. Primers can incorporate additional features which allow for the detection or immobilization of the primer but do not alter the basic property of the primer, that of acting as a point of initiation of DNA synthesis. The term "primer", as used herein, also refers to a set of oligonucleotides which provide sufficient sequence variants of the
30 hybridization region to permit hybridization with each member of a given set of target sequence variants, so as to act as a point of initiation of DNA synthesis. Additionally, a primer may consist of one or more oligonucleotides which contain mismatches with some or all members of a given set of target sequence variants, but contains sufficient

regions of complementarity with each target sequence variant so as to enable hybridization with all target sequence variants under suitable conditions. The term "consensus primers" is used herein to refer to primers containing a single oligonucleotide complementary to a consensus target sequence, to primers consisting of multiple oligonucleotides complementary to a consensus target sequence, and to combinations thereof.

Samples of the patient's tissue or body fluids suitable for the use in the detection method using probes include those which are discussed above with regard to detection methods employing antibodies.

Amplification of breast tissue specific nucleic acid molecules (i.e., nucleic acid molecules encoding the chemokines of the present invention) is preferably carried out by PCR. Use of PCR to amplify DNA is described in U.S. Patent. No. 4,683,195 to Mullis et al., U.S. Patent. No. 4,683,202 to Mullis, and U.S. Patent. No. 4,965,188 to Mullis et al., which are hereby incorporated by reference. Briefly, PCR amplification of DNA involves repeatedly heat-denaturing the DNA, annealing two oligonucleotide primers to sequences that flank the DNA segment to be amplified, and extending the annealed primers with DNA polymerase. The primers hybridize to opposite strands of the target sequence and are oriented so DNA synthesis by the DNA polymerase proceeds across the region between the primers, effectively doubling the length of that DNA segment. Moreover, because the extension products are also complementary to and capable of binding primers, each successive cycle essentially doubles the amount of DNA synthesized in the previous cycle. This results in the exponential accumulation of the specific target fragment at a rate of approximately 2^n , where n is the number of cycles. Due to the enormous amplification possible with the PCR process, small levels of DNA carryover from samples with high DNA levels can result in PCR product, even in the absence of purposefully added template DNA. Optimally, all reaction mixes are set up in an area separate from PCR product analysis and sample preparation and care is taken to avoid cross contamination, for example, by using dedicated or disposable vessels, solutions, pipettes (preferably positive displacement pipettes), and pipette tips (preferably with aerosol barriers) for RNA/DNA, reaction mixing, and sample analysis. See e.g., Higuchi et al., Nature 339:237-238 (1989) and Kwok et al. in Innis et al., eds., PCR Protocols: A Guide to Methods and Applications, San Diego, California: Academic Press, Inc., pp. 142-145 (1990), which are incorporated herein by reference.

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Primers suitable for use in the method of the present invention are preferably 15 to 30 nucleotides in length and are designed to have a high degree of homology with breast tissue specific nucleic acid sequences (i.e., with nucleic acid molecules encoding chemokines of the present invention). For each region to be amplified, two regions of homology are required, one for negative-strand primers and another for positive-strand primers. Once a homologous region is identified, a consensus primer is designed. Degenerate bases can be used in the design to accommodate positions at which an individual breast tissue gene varies in sequence from the consensus sequence (genetic polymorphism). Preferably, as many degenerate positions are made as is necessary so that all breast tissue sequences have fewer than three mismatches with the consensus primer. Any mismatches that are not accommodated by the degenerate positions in the primer should preferably be located more than 3 bases from the 3' end of the primer. Likewise, any degenerate positions should preferably be more than 3 bases from the 3' end of the primer. Degenerate primers having estimated minimum and maximum Tms of about 54 °C and about 64 °C, respectively, are preferred, where Tms are estimated by summing a contribution from each base pair. In this formulation, each G or C contributes 4 °C to the Tm, and each A or T contributes 2 °C to the Tm. Finally, it is generally preferred that primers be designed so that they do not span palindromes or repetitive sequences.

Following amplification, the breast tissue specific nucleic acid molecules are detected to determine whether amplification has occurred. Since amplification will occur (and breast tissue specific nucleic acid molecules will be detected) only if some amount of breast tissue specific nucleic acid molecules were present in the sample before amplification, detection of breast tissue specific nucleic acid molecules after amplification indicates the presence of breast disease in the patient from which the sample came.

Suitable nucleic acid primers include those which, under stringent hybridization conditions, hybridize to a nucleic acid molecule encoding a chemokine having an amino acid sequence corresponding to SEQ. ID. No. 1 and/or which hybridize to a nucleic acid molecule having a nucleotide sequence corresponding to SEQ. ID. Nos. 6-8. In particular, suitable nucleic acid primers include those having a nucleotide sequence corresponding to SEQ. ID. No. 9 or SEQ. ID. No. 10.

There are a variety of known methods for determining whether amplification has occurred. For example, a portion of the PCR reaction mixture can be subjected to gel electrophoresis, the resulting gel can be stained with, for example, a ultraviolet absorbing stain, such as with ethidium bromide, and the stained gel can be exposed to ultraviolet light to determine whether a product of the expected size can be observed. Alternatively, labeled PCR primers or labeled deoxyribonucleoside 5'-triphosphates can be used to incorporate the label into the amplified DNA. The presence of a breast tissue specific nucleic acid amplification product can then be detected by detecting the label. Examples of suitable labels and label detection methods include those set forth above with regard to the detection method which employed hybridization. Another method for determining if amplification has occurred involves testing a portion of the amplified reaction mixture for ability to hybridize to a labeled probe designed to hybridize only to the amplified DNA. Amplified breast tissue specific nucleic acid molecules can also be detected by DNA sequencing as well as by microscopic visualization.

A number of treatments can be used to amplify the breast tissue specific nucleic acid molecules (i.e., nucleic acid molecules encoding a chemokine of the present invention). These include PCR, ligase chain reaction ("LCR"), self-sustained sequence ("3SR") replication, Q-beta replicase, nucleic acid sequence based amplification ("NASBA"), transcription-based amplification System ("TAS"), or branched-DNA methods.

Although PCR is the preferred amplification method, amplification of target sequences in a sample may be accomplished by any known amplification method, such as ligase chain reaction methods (described, e.g., in Wu et al., Genomics 4:560-569 (1988), which is hereby incorporated by reference). In LCR, the consensus primers can be used to direct the joining of oligonucleotide segments that anneal to the target nucleic acid, thereby amplifying the target. Further details with regard to this method can be found in, for example, WO 89/09835, which is hereby incorporated by reference. Other suitable amplification methods include the TAS amplification system (described, e.g., in Kwoh et al., Proc. Natl. Acad. Sci. USA 86:1173-1177 (1989), which is hereby incorporated by reference), branched-DNA methods (described, e.g., in Kern et al., J. Clin. Microbiol. 34:3196-3202 (1996), which is hereby incorporated by reference), and self-sustained sequence replication methods (described, e.g., in Guatelli et al., Proc. Natl.

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Acad. Sci. USA 87:1874-1878 (1990), which is hereby incorporated by reference). Each of these methods provides sufficient amplification so that the target sequence can be detected by nucleic acid hybridization to an oligonucleotide probe, such as those described above, or by other detection methods. Alternatively, methods that amplify the probe to detectable levels, such as Q-beta replicase amplification can be employed. This method is described in, for example, Kramer et al., Nature 339:401-402 (1989) and Lomeli et al., Clin. Chem. 35:1826-1831 (1989), which are hereby incorporated by reference. Further details regarding these and other suitable amplification methods are provided in Abramson et al., Current Opinion in Biotechnology 4:41-47 (1993), which is hereby incorporated by reference. The term "probe", as used with regard to the above amplification methods, encompasses any of the sequence-specific oligonucleotides used in these procedures. For instance, the two or more oligonucleotides used in LCR are "probes" for purposes of the present invention, even though some embodiments of LCR only require ligation of the probes to indicate the presence of an allele.

In some cases, the tissue or fluid sample from the patient may contain a breast tissue specific nucleic acid transcript (i.e., mRNA) which codes for the chemokine of the present invention. In this situation, the mRNA can be converted to cDNA by reverse transcription-PCR ("RT-PCR") prior to amplification. This involves treating the mRNA-containing sample with reverse transcriptase in an appropriate reaction mixture and in the presence of an appropriate primer. The primer used in the reverse transcription reaction can be a consensus primer of the present invention, or it can be a different oligonucleotide that hybridizes near the 3' end of the mRNA. Although random hexamers are not specific for the 3' end of the mRNA molecule, they are suitable for reverse transcription of mRNA to provide a cDNA template for amplifying breast tissue specific nucleic acids. This cDNA copy is then made into a double stranded DNA molecule, which can be amplified as described above.

The nucleic acid primer used in the above amplification detection method may be assembled as a kit for detecting breast disease. Such a kit includes consensus primers and molecular probes. A preferred kit also includes the components necessary to determine if amplification has occurred. The kit may also include, for example, PCR buffers and enzymes; positive control human breast tissue specific sequences, reaction control primers, such as betaglobin primers; and instructions for amplifying and detecting breast tissue specific sequences.

The symbols used herein to designate particular nucleotides are set forth below in Table 2.

TABLE 2

5

	<u>Symbol</u>	<u>Meaning</u>
	G	guanine
	A	adenine
10	T	thymine
	C	cytosine
	R	adenine or guanine
	Y	cytosine or thymine
	M	adenine or cytosine
15	K	guanine or thymine
	S	cytosine or guanine
	W	adenine or thymine
	H	adenine or cytosine or thymine
	B	cytosine or guanine or thymine
20	V	adenine or cytosine or guanine
	D	adenine or guanine or thymine
	N	adenine or cytosine or guanine or thymine

The present invention is further illustrated by the following examples.

25

EXAMPLES

Example 1 -- Isolation of Novel Human Breast Tissue Specific Nucleic Acid Sequences Using Suppression Subtractive Hybridization

30

Suppression Subtractive Hybridization ("SSH") was performed according to the protocol of Diatchenko et al., Proc. Natl. Acad. Sci. USA 93:6025-6030 (1996), which is hereby incorporated by reference, using commercial reagents from Clontech (PCR-Select cDNA subtraction kit). Human polyA RNAs derived from bone marrow, skeletal muscle, lung, liver, pancreas, and mammary gland were obtained from Clontech,

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and 2 mg of each were reverse transcribed. The cDNAs derived from mammary gland were subdivided and ligated to different cDNA adaptors according to the manufacturer's protocol. Primary and secondary subtractive hybridizations were performed by adding an excess of denatured cDNAs derived from human bone marrow, lung, pancreas, liver, and skeletal muscle ("driver" cDNAs) to the mammary gland cDNA ("tester cDNA"). The entire population of subtracted molecules was subjected to two rounds of DNA amplification: a primary PCR to amplify differentially expressed sequences and a secondary (nested) PCR to enrich for those sequences. PCR primers 1 and 2 and nested PCR primers 1 and 2 (Clontech) were used in accordance with the protocol of the PCR-Select cDNA subtraction kit for primary and secondary PCR, respectively. All DNA amplifications were performed with a Perkin-Elmer DNA Thermal Cycler Model 2400 using parameters of 94 °C, 5 seconds (denature); 68 °C, 30 seconds (anneal); and 72 °C, 150 seconds (extend) and using the Advantage KlenTaq Polymerase Mix (Clontech) which contains a TaqStart Antibody to provide automatic hot start PCR (Kellogg et al., Biotechniques 16:1134-1137 (1994), which is hereby incorporated by reference). PCR was optimized using the control reagents contained in the PCR-Select cDNA subtraction kit as template and the OPTI-PRIME™ PCR Optimization Kit (Stratagene). Amplification products were analyzed by gel electrophoresis (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press (1987) ("Sambrook") and Ausubel et al., Current Protocols in Molecular Biology, New York: Greene Publishing Associates and Wiley-Interscience (1990) ("Ausubel"), which are hereby incorporated by reference). In our hands, the optimal buffer for primary PCR contained 40 mM Tricine-KOH (pH 9.2), 15 mM KOAc, 3.5 mM Mg(OAc)₂, and 75 mg/ml bovine serum albumin (10X KlenTaq PCR reaction buffer, Clontech). The optimal buffer for secondary PCR contained 10 mM Tris-HCl (pH 8.3), 75 mM KCl, and 3.5 mM MgCl₂ (Stratagene, Opti-Prime 1x Buffer #4) with 5% dimethylsulfoxide.

Example 2 -- Cloning of the Subtracted cDNAs

Nested PCR primer 1 was phosphorylated using reagents from Invitrogen (Eukaryotic TA Cloning Kit, Unidirectional). Secondary PCR (10 cycles) was performed in the optimized buffer described above using nested PCR primer 2 and the phosphorylated nested PCR primer 1. PCR products were directionally ligated into the

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mammalian expression TA cloning vector pCRTM3.1-Uni and transformed into TOP10F' competent cells using general techniques (Sambrook and Ausubel, which are hereby incorporated by reference) and commercial reagents from InVitrogen. pCRTM3.1-Uni contains a T-overhang which allows the direct cloning of PCR products containing single
5 3' A-overhangs (Mead et al., Bio/Technology 9:657-663 (1991), which is hereby incorporated by reference. Transformed cells were selected in Luria-Broth media containing 25 mg/ml kanamycin.

Example 3 -- Sequencing of Differentially Expressed Clones

10 DNA plasmid isolations were performed using the Qiagen Plasmid Mini Kit which employs the alkaline lysis method (Sambrook, which is hereby incorporated by reference). Plasmids were screened for insert sequences using nested PCR primers 1 and 2 and the protocol and reagents from the Geneamp PCR Kit (Perkin Elmer), and amplified products were analyzed by gel electrophoresis. Clones containing inserts
15 greater than 100 basepairs ("bp") were obtained for sequencing analysis. Dideoxy DNA sequencing was performed using the Applied Biosystems Model 373 Automated DNA Sequencing System. The DNA sequence of each strand was determined using sequencing primers T7 (5' TAATACGACTCACTATAGGG 3') (SEQ. ID. No. 12) and pCRTM3.1 Reverse (5' TAGAAGGCACAGTCGAGG 3') (SEQ. ID. No. 13), respectively.

20

Example 4 -- Search for Genetic Homologies

GenBank was searched for homologous sequences via the program BLASTN (Altschul et al., J. Mol. Biol. 215:403-410 (1990) and Benson et al., Nucleic Acids Res. 24:1-5 (1996), which are hereby incorporated by reference). Sequences were
25 classified as known or unknown based on the resulting score and probability values. Known sequences were arbitrarily defined as those having probability values greater than 0.05 ($p > 0.05$) relative to database sequences or those showing homology to non-human species or to cosmids containing human DNA of which a function has not been assigned.

Example 5 -- Rapid Amplification of cDNA Ends

30 Full length mammary associated chemokine ("MACK") cDNA was generated using 5' and 3' rapid amplification of cDNA ends ("RACE") (Frohman, PCR Protocols, New York:Academic Press, pp. 28-39 (1990), which is hereby incorporated by reference) using commercial reagents (Marathon cDNA Amplification Kit, Clontech).

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Human mammary gland polyA RNA (Clontech) was used as a template for first and second strand cDNA synthesis, and adaptors were ligated to the pool of cDNA according to the manufacturer's protocol. The 3' RACE product was obtained by using the gene-specific primer (24R) 5' ACTGGGATTATAGGTGTGAGCC 3' (SEQ. ID. No. 10) and Clontech's adaptor primer 1 (AP1) using "Touchdown PCR" according to the manufacturer's directions. This was followed by a secondary PCR using the nested gene-specific primer (24R2) 5' CAAATTCAGGTTTAATGTTTTTGG 3' (SEQ. ID. No. 14) and Clontech's nested adaptor primer 2 (AP2). PCR products were cloned into the T/A cloning vector pCR2.1 (Invitrogen). DNA plasmid preparations were prepared and sequenced using vector sequences T7 and M13 reverse. Internal sequencing primers were based on confirmed sequences.

The 5' RACE product was obtained using Clontech's MARATHON™ Ready cDNA from human mammary gland according to their protocol. "Touchdown PCR" was performed on the cDNA using gene-specific primer (F4) 5' CTCAAACGTGTGAGCCCCGCA 3' (SEQ. ID. No. 15) and AP1, and nested PCR was performed using nested gene-specific primer (F3) 5' GCTACTCAAACCTAGACGTTTTGAAG 3' (SEQ. ID. No. 16) or (F1) 5' GAATTCACGTAGGAAATTCTTAACC 3' (SEQ. ID. No. 9) and AP2 (see above). PCR products were cloned and sequenced as described above. A consensus sequence was generated using programs from the Hitachi software package DNAsis for Windows.

Example 6 -- Northern Blot Analysis

Human mammary gland PolyA+ RNA (3 µg, Clontech Laboratories, Inc.) was separated and transferred using the NORTHERNMAX™ Northern Blotting Kit from Ambion. PCR amplification of a 302 bp region within the predicted ORF was performed using primers F8 5' CCGTATGTTTCGTGTTTCCCCTGA 3' (SEQ. ID. No. 17) and R5 5' AGCCATACTTCCCATTCCTCCAG 3' (SEQ. ID. No. 18) and 5' RACE clone (#27) as template. This fragment was directionally ligated to a T7 promoter (LIG'NSCRIBE™ RNA Polymerase Promoter Addition Kit, Ambion) and amplified such that the antisense strand was orientated immediately downstream to the T7 promoter according to the manufacturer's protocol. An antisense riboprobe having SEQ. ID. No. 11 was transcribed *in vitro* using T7 RNA polymerase, and labeled using the BRIGHTSTAR™ Psoralen-Biotin Nonisotopic Labeling Kit (Ambion). Hybridization

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and chemiluminescent detection were performed using protocols from Ambion's NORTHERNMAX™ and BRIGHTSTAR™ BIODETECT™ Kits, respectively.

Example 7 -- Production of Antisera to the Open Reading Frame Protein Sequence

5 The predicted open reading frame within the MACK gene was determined using commercial software (DNAsis, Hitachi Corp.). Synthetic peptides corresponding to predicted immunogenic domains, KLH-peptide conjugates and resultant rabbit antisera were produced by Research Genetics, Inc. (Huntsville, AL). Antisera were collected after a 10-week immunization protocol.

10

Example 8 -- Titration of Anti-peptide Antisera

 Synthetic peptides were dissolved in 0.2 M carbonate-bicarbonate buffer, pH 9.4 (CBC buffer) at a concentration of 10 µg/mL. Microplates were coated (100 µL/well) with the peptides at 4 °C for 18 hrs. The solution was removed and the
15 microwells were blocked with 1% bovine serum albumin in tris-buffered saline ("TBS"), pH 7.4 for 1 hr. Dilutions of anti-peptide antisera were incubated with the solid-phase peptides for 1 hr, and, following a wash procedure, goat antibodies to rabbit immunoglobulin (biotin-conjugated) were added for 30 min. After another wash procedure, each well received 100 µL of avidin-biotinylated alkaline phosphatase
20 complex (ABC Kit, Pierce Immunochemicals) for 30 min. Thereafter, the wells were washed, and substrate (para-nitrophenyl phosphate, 1 mg/ml in diethanolamine buffer, pH 9.8) was added for 30 min. After stopping the reactions with 50 µL of 5 N NaOH, optical density was determined at an absorbance of 450 nm using a microplate spectrophotometer.

25 Example 9 -- Purification of IgG and Enzyme Coupling

 IgG from rabbit serum was purified using protein A affinity chromatography (MAPS II Kit, Bio-Rad Labs). IgG was conjugated to horseradish peroxidase using the periodate oxidation technique (Nakane et al., J. Histochem. Cytochem. 22:1084-1091 (1974), which is hereby incorporated by reference).

30

Example 10 -- SDS-PAGE and Western Blotting

 SDS-PAGE was performed as described in Laemmli, Nature 227:680-685 (1970) ("Laemmli"), which is hereby incorporated by reference.

Western blotting was performed essentially as described in Papsidero et al., *Hybridoma* 7:117-128 (1988), which is hereby incorporated by reference, using nitrocellulose paper with a 0.22 μ m pore size. Blots were incubated for 1 hr at room temperature with immune or pre-immune sera diluted in assay buffer. The membranes were washed and developed with avidin-biotin-alkaline phosphatase reagents using commercial reagents (ABC Kit, Pierce Immunochemicals). Blots were developed with insoluble substrate (BCIP/NBT solution, Pierce Immunochemicals), washed in water and air-dried.

10 Example 11 -- Results of Comparison of Isolated Sequence Tags to GenBank

Human breast tissue mRNA was subjected to SSH and 118 sequence tags were isolated and sequenced. Of the total examined, 62% (73 of 118) were homologous to genes found in the GenBank database (Table 3). Of interest, approximately 14% (10 of 73) of the previously described sequences were breast tissue specific or highly associated with breast tissue (i.e., casein isoforms, alpha-lactalbumin, and milk fat globule proteins). Remarkably, 38% of the sequence tags (45 of 118) demonstrated no significant homology with genes found in the database (Table 3). These novel genes were studied further using RT-PCR in order to determine the specificity of their tissue expression.

TABLE 3
Human Breast Tissue mRNA Sequence Tags Isolated
Using Suppression Subtraction Hybridization

ID #	GenBank Search	insert size (bp)	Strongest Homology	Blast Score (probability)	Identical residues/Total residues (%)
1	Known	309	Human keratin	459 (p<0.001)	99 / 108 (91%)
5	Known	195	Human A1S9 mRNA	619 (p<0.001)	127 / 133 (95%)
7	Known	66	Human Vimentin	330 (p<0.001)	66/66 (100%)
8	Unknown	198	<i>S. cerevisiae</i>	114 (p=1.0)	30 / 39 (76%)

10	Known	96	H. sapiens rho GAP protein	462 (p<0.001)	95/98 (96%)
11	Known	105	Mouse cerbA alpha 2 mRNA (thyroid H.)	507 (p<0.001)	105/105 (100%)
14	Known	135	TCR eta = Tcell receptor eta chain	258 (p<0.001)	62 / 75 (82%)
16	Known	115	Pancreatic peptidylglycine	557 (p<0.001)	113 / 115 (98%)
20	Known	182	H. sapiens paraoxynase	520 (p<0.001)	122 / 146 (83%)
22	Known	194	Human mRNA for cytoskeletal gamma actin	956 (p<0.001)	192/194 (99%)
23	Unknown	201	Chimpanzee cmyc protooncogene	134 (p=0.18)	42 / 61 (68%)
28	Known	150	Milk fat globule protein (human)	515 (p<0.001)	103 / 103 (100%)
30	Known	442	H. sapiens mitochondrial genome	1245 (p<0.001)	251 / 254 (98%)
47 & 67	Unknown	143	Beet necrotic yellow vein virus	134 (p=0.10)	54 / 88 (61%)
51	Known	174	H. sapiens mRNA homologue to yeast ribo. Protein	831 (p<0.001)	169 / 174 (97%)
54	Unknown	125	M. musculus for Notch 3	179 (p<0.001)	45 / 57 (78%)
57	Known	180	H. sapiens cDNA for betacasein	715 (p<0.001)	147 / 155 (94%)
60	Unknown	202	X. laevis mRNA for DNA binding	122 (p=0.88)	42 / 64 (65%)
61	Known	286	Human 28k basic protein	1349 (p<0.001)	273 / 278 (98%)
62	Known	195	Human A1S9 mRNA	968 (p<0.001)	194 / 195 (99%)
74	Known	152	Human MER 37 transposable element	351 (p<0.001)	87/108 (80%)
75	Known	192	Human mRNA for cytoskeletal gamma actin	960 (p<0.001)	192 / 192 (100%)
78	Unknown	626	C. elegans ZK1073	123 (p=1.0)	31 / 39 (79%)

79 & 80	Unknown	90 & 100	Myxococcus xanthus photolyase	113 (p=0.96)	29 / 37 (78%)
82	Unknown	295	Actinobacillus riboflavin biosynthesis operon	121 (p=0.99)	41 / 62 (66%)
89	Known	214	Human casK mRNA for Kappa casein	1063 (p<0.001)	213 / 214 (99%)
101	Unknown	99	C. elegans C35B8	118 (p=0.71)	34 / 47 (72%)
105	Known	84	H. sapiens mRNA for 90K product	357 (p<0.001)	75 / 84 (89%)
114	Unknown	111	Human peregrin mRNA	127 (p=0.23)	39 / 56 (69%)
115	Known	186	Rat 8s RNA	728 (p<0.001)	147 / 151 (97%)
116	Unknown	413	M. musculus for p38264	787 (p<0.001)	171 / 190 (99%)
120	Known	253	Human SF 2 p33 mRNA (splicing factor)	1223 (p<0.001)	247 / 253 (97%)
121	Unknown	154	M. musculus serum inducible	653 (p<0.001)	141 / 154 (91%)
122	Unknown	354	Drosophila silver p.	264 (p<0.001)	118 / 202 (58%)
127	Known	133	H. sapiens mRNA for rat HREV 107like	368 (p<0.001)	96 / 125 (76%)
131	Unknown	117	C. elegans R12C12	126 (p=0.31)	38 / 54 (70%)
133	Unknown	133	Bos taurus polymeric immunoglobulin	149 (p<0.001)	33 / 37 (89%)
135	Unknown	124	Rat vesicle associated membrane protein	286 (p<0.001)	60 / 64 (93%)
140	Known	312	Human ferritin	1530 (p<0.001)	308/312 (98%)
142	Unknown	123	Human MAGE 4a antigen gene	129 (p=0.21)	37 / 51 (72%)
143	Known	94	Human ribosomal protein L28	470 (p<0.001)	94/94 (100%)
145	Unknown	283	M. auratus beta myosin	132 (p=0.39)	52 / 84 (61%)

152	Known	551	H. sapiens mitochondrial genome	751 (p<0.001)	153 / 157 (97%)
155	Unknown	238	R. norvegicus adenyl cyclase	109 (p=0.87)	35 / 52 (67%)
158	Known	186	Rat 8s RNA	698 (p<0.001)	142/146 (97%)
162	Known	129	Gamma actin	629 (p<0.001)	127/129 (98%)
164	Known	95	Human mRNA for OSF1	452 (p<0.001)	92 / 95 (96%)
171	Known	321	Human mRNA for cytokeratin	1033 (p<0.001)	209/213 (98%)
175	Unknown	134	M. musculus isocitrate dehydrogenase	130 (p=0.19)	36 / 49 (73%)
176	Known	150	Human mitochondrial DNA	750 (p<0.001)	150/150 (100%)
178	Unknown	269	Gorilla ALU repeat / H. sapiens casein kinase	191 (p<0.001)	47 / 60 (78%)
179	Known	182	Human CORE1 protein	903 (p<0.001)	181 / 182 (99%)
181	Known	155	Human alphalactalbumin	712 (p<0.001)	144 / 147 (97%)
182 & 197	Unknown	259	Human DNA sequence from cosmid N28H9	196 (p<0.001)	78 / 127 (61%)
188	Known	216	Human ALU	453 (p<0.001)	101/114 (88%)
189	Unknown	105	Human DNA sequence from cosmid N37F	125 (p<0.001)	31 / 39 (79%)
192	Unknown	104	M. musculus cytoplasmic protein	119 (p=0.62)	27 / 31 (87%)
195	Known	155	Human alphalactalbumin	696 (p<0.001)	144/147 (97%)
196	Known	156	Mouse 28s rRNA	412 (p<0.001)	84/86 (97%)
201	Known	183	Human CORE1 protein	841 (p<0.001)	169 / 171 (98%)
204	Unknown	194	Human DNA sequence from cosmid L139H	514 (p<0.001)	118 / 138 (85%)

205	Known	54	Human cytokeratin	238 (p<0.001)	48/49 (97%)
207	Known	139	Human prostaticin	589 (p<0.001)	119 / 121 (98%)
208	Unknown	356	Human cathepsin D (catD) gene	130 (p=0.64)	34 / 44 (75%)
209	Known	373	Putative zinc finger Rattus norvegicus	707 (p<0.001)	161 / 195 (82%)
210	Known	129	Gamma actin	606 (p<0.001)	124/129 (97%)
214	Known	105	Alphalactalbumin	509 (p<0.001)	103/105 (98%)
216	Known	153	Alphalactalbumin	709 (p<0.001)	143/145 (98%)
218	Known	190	Acidic calponin	941 (p<0.001)	189 / 190 (99%)
220	Unknown	99	C. elegans cosmid C34E7	108 (p=1.0)	28 / 36 (77%)
221	Unknown	122	S. cerevisiae chromosome	121 (p=0.33)	22/25 (87%)
223	Unknown	91	Bovine betahydroxylase	113 (p=0.94)	29 / 37 (78%)
224	Known	164	Lactate dehydrogenase	614 (p<0.001)	124/127 (97%)
225	Known	273	Proalpha collagen	1335 (p<0.001)	269/273 (98%)
229	Known	235	Collagen	1143 (p<0.001)	232/235 (98%)
230	Unknown	117	Plasmodium falciparum (strain FCR3)	116 (p=0.89)	30 / 39 (76%)
231 & 234	Unknown	94	CNS myelin P0like glycoprotein	124 (p=0.26)	40 / 59 (67%)
232	Unknown	405	H. sapiens mRNA for 218kD Mi2 protein	132 (p=0.55)	42 / 62 (67%)
233	Unknown	198	Rat TnT gene encoding troponin T	130 (p=0.36)	34 / 44 (77%)
238	Known	140	Human Thy 1 glycoprotein	645 (p<0.001)	133 / 140 (95%)

242	Known	136	H. sapiens casK mRNA for Kappa casein	666 (p<0.001)	134/136 (98%)
249	Known	136	H. sapiens casK mRNA for Kappa casein	680 (p<0.001)	136/136 (100%)
250	Known	288	H. sapiens CpG DNA	792 (p<0.001)	164/172 (95%)
252	Known	525	Human pHL1 gene (cmv oncogene)	1704 (p<0.001)	352/377 (93%)
253	Known	125	Human mRNA for plasma gelsolin	618 (p<0.001)	124 / 125 (99%)
255	Known	138	Human Xq 28 genomic DNA	333 (p<0.001)	69 / 74 (93%)
256	Known	56	Human vimentin	280 (p<0.001)	55/55 (100%)
257	Known	236	Human breast cancer LIV1 regulated mRNA	1134 (p<0.001)	230 / 236 (97%)
258	Known	125	Human gelsolin	618 (p<0.001)	124/125 (99%)
261	Known	283	Human mRNA for ORF myeloblast cellline	1394 (p<0.001)	280 / 283 (98%)
263	Known	156	Human pemphigoid autoantigen	773 (p<0.001)	155 / 156 (99%)
264	Unknown	198	C. elegans N2 basic helix	116 (p=0.99)	36 / 52 (69%)
269	Unknown		No Matches Identified	N/A	N/A
275	Known	283	Human mRNA for ORF	1373 (p<0.001)	277 / 283 (97%)
276	Unknown	195	C. elegans cosmid ZK813	133 (p=0.20)	41 / 59 (69%)
279	Known	339	Alpha casein	1674 (p<0.001)	336 / 339 (99%)
284	Known	129	H. sapiens BTF2p44 mRNA for basic transcription	645 (p<0.001)	129 / 129 (100%)
287	Known	293	Human mRNA	1251 (p<0.001)	261 / 280 (93%)
291	Unknown	171	D. melanogaster chromosome 3 locus 85D	133 (p=0.18)	33 / 41 (80%)

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292	Known	148	H. sapiens HIV1 TAR RNA binding protein	699 (p<0.001)	143 / 148 (96%)
297	Known	136	Human migration inhibitory factor mRNA	617 (p<0.001)	127 / 136 (93%)
300	Unknown	176	R. norvegicus FSHregulated protein mRNA	427 (p<0.001)	91 / 98 (92%)
302	Unknown	96	S. platensis rpsB gene (ribosomal protein S2)	111 (p=0.99)	43 / 69 (62%)
303	Known	146	H. sapiens alphasalalbumin	705 (p<0.001)	141 / 141 (100%)
305	Known	99	B. taurus myosin IB mRNA	336 (p<0.001)	80 / 99 (80%)
308	Unknown	295	D. melanogaster Oregon R mRNA	422 (p<0.001)	134 / 197 (68%)
314	Unknown	158	Maize mRNA for catalase 2	113 (p=1.0)	29 / 37 (78%)
329	Unknown	160	C. elegans cosmid C09B9	117 (p=0.97)	39 / 59 (66%)
330	Known	109	Human nonmuscle myosin alkali light chain	531 (p<0.001)	107 / 109 (98%)
333	Unknown	119	Mouse MA3 (apoptosisrelated gene) mRNA	124 (p=0.39)	30 / 37 (81%)
337	Unknown	99	No Matches Identified	N/A	N/A
338	Unknown	271	Human fur gene, exons 1 through 8	143 (p=0.057)	51 / 79 (64%)
339	Known	65	H. sapiens mRNA for IgG1 heavy chain	123 (p=0.012)	35 / 48 (72%)

At least one expressed sequence tag (Table 1, ID # 189), designated Breast Sequence Tag-24 (BRST-24"), was demonstrated to exhibit a high level of specificity to breast tissue. BRST-24 has SEQ. ID. No. 8 as follows:

5

ACACGAATTCACGTAGGAAATTCTTAACCAAAAACATTAAACCTGAATTTGA
TCACAAGAAAATAATTAGGCCAGGCACTGTGGCTCACACCTATAAATCCCAGT

Example 12 -- Tissue Specificity Analysis of BRST-24 Using RT-PCR

The tissue specificity of BRST-24 was experimentally demonstrated using RT-PCR analysis of various human tissue mRNAs along with primers which are complementary to regions of the BRST-24 nucleotide sequence. The primers had the following sequences:

GAATTCACGTAGGAAATTCTTAACC (F1 primer)

ACTGGGATTATAGGTGTGAGCC (R1 primer)

These sequences are respectively identified herein as SEQ. ID. No. 9 and SEQ. ID. No. 10.

Example 13 -- Detection of BRST-24 Using RT-PCR Analysis of Human Tissues

RT-PCR was performed using the protocol and reagents from the Perkin-Elmer GeneAmp EZ rTth RNA PCR Kit. PCR primers BRST-24 fwd (5' GAATTCACGTAGGAAAT TCTTAACC 3') (SEQ. ID. No. 9) and BRST-24 rev (5' ACTGGGATTATAGGTGTGAGCC 3') (SEQ. ID. No. 10) were synthesized by Research Genetics. A tissue panel of total RNAs derived from human testis, brain, lung, prostate, kidney, skeletal muscle, small intestine, liver, pancreas, uterus, and breast (all obtained from Clontech) was screened via RT-PCR for the presence of BRST-24 using a Perkin-Elmer DNA Thermal Cycler Model 2400. Reverse transcription was carried out for 30 minutes at 60 °C, the reaction mix was denatured at 94 °C for one minute followed by 40 cycles of PCR (94 °C, 15 seconds (denature), 60 °C, 30 seconds (anneal and extend)), and a final extension was carried out for 7.0 minutes at 60 °C. The amplified products were observed on a 3% agarose gel (0.5X TBE) as described in Sambrook, which is hereby incorporated by reference.

As shown in Table 4, the BRST-24 primer pair was able to be utilized to amplify nucleotide sequences from all of three specimens of human breast tissue mRNA using RT-PCR. These specimens included two normal breast tissue pools and one specimen of invasive ductal carcinoma. Other human tissue mRNAs examined were noted to contain no detectable, amplifiable mRNA genetic sequences corresponding to BRST-24. These tissues included liver, lung, small intestine, pancreas, uterus, brain, kidney, and skeletal muscle. A testes specimen did, however, produce a faint reaction product. As an experimental control, mRNA sequences specific for prostate specific

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antigen ("PSA") were detected by RT-PCR using primers homologous to regions within the PSA nucleic acid sequence (Deguchi et al., Cancer Research 53:5350-5354 (1993), which is hereby incorporated by reference). As seen in Table 4, PSA mRNA was exclusively detected in human prostate tissue, confirming the specificity of the PSA mRNA expression and the integrity of the experimental protocol.

TABLE 4
Differential Expression of BRST-24 and PSA Transcripts
in Human Tissues as Detected Using RT-PCR

Tissue	Normal/ Malignant	BRST-24 ⁴ Expression	PSA ⁵ Expression
Breast ¹	Normal	2+ ⁶	ND ⁷
Breast ²	Normal	2+	-
Breast ³	Carcinoma	2+	ND
Prostate	Normal	-	2+
Kidney	Normal	-	-
Pancreas	Normal	-	-
Small Intestine	Normal	-	-
Skeletal Muscle	Normal	-	-
Testis	Normal	+/-	-
Brain	Normal	-	-
Uterus	Normal	-	-
Liver	Normal	-	-
Pancreas	Normal	-	-

1) Human mammary gland poly A⁺ RNA isolated from a pool of 4 specimens (Caucasian, ages 34-49).

2) Human mammary gland total RNA isolated from a pool of 6 specimens (Caucasian, ages 16-35).

3) Total RNA isolated from an invasive ductal carcinoma of the breast (Asian, age 36).

4) RT-PCR using primer pair specific for BRST-24 (SEQ. ID. Nos. 8 and 9)

5) RT-PCR using primer pairs specific for Prostate Specific Antigen (Deguchi et al., Cancer Research 53:5350-5354 (1993), which is hereby incorporated by reference).

6) -, negative; +/-, equivocal; 1+, weak; 2+, strong reaction product.

7) ND, not done.

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Expression of BR-24 transcript was also monitored using Northern blotting with an internal probe from the BR-24 cDNA sequence having a sequence corresponding to SEQ. ID. No. 11.

Northern blot analysis of polyA RNA from human mammary gland
 5 resulted in the detection of a transcript appearing slightly above the 3000 base pair marker. This is consistent with the predicted transcript size based upon results from RACE construction of the full-length cDNA.

BR-24 nucleic acid sequences were also detected in human cell lines using RT-PCR along with the same primers used in the above experiments. Results as, seen in
 10 Table 5, provide additional support to the view that the BR-24 gene is expressed preferentially in human mammary cells.

15
 TABLE 5
 Detection of BR-24 Transcripts in
 Cultured Human Cell Lines

Cell Line	Description	Expression of BR-24 Transcripts
BT-20	Breast Carcinoma	2+
MCF-7	Breast Carcinoma	1+
MDA-MB-157	Breast Carcinoma	-
SK-OV-3	Ovary Carcinoma	-
LNCaP	Prostate Carcinoma	-
SW620	Colon Carcinoma	1+-

20 Example 14 -- Isolation of the Full-length BR-24 cDNA

To obtain the full-length cDNA sequence of MACK, the 5' and 3' RACE clones were overlapped. Thus, this sequence represents the consensus of 5' and 3' RACE clones from a population of donor mRNAs. The 5' RACE clones varied in length at the 5' end which may be attributed to secondary structure and pausing of the reverse
 25 transcription during cDNA synthesis. Using this method, a consensus cDNA sequence

of 3117 base pairs, excluding the polyA tail was generated. This sequence is identified herein as SEQ. ID. No. 6.

Using computer algorithms (DNASIS software package, Hitachi Corp.), the open reading frame was determined to encode a protein of 127 amino acids, between
5 nucleic acid bases 47 and 428 above. The amino acid sequence of this protein is identified herein as SEQ. ID. No. 1. The deduced molecular weight of the protein was 14,232 daltons, and the deduced isoelectric point was pH 10.44.

Of interest, the above protein sequence shared sequence homology with a class of cytokines designated as "chemokines" (See Baggiolini et al., Ann. Rev. Immunol.
10 15:675-705 (1997) and Rollins, Blood 90:909-928 (1997), which are hereby incorporated by reference. Thus, the above sequence represents a new member of the "CC" or "β" class of chemokines. Figure 1 shows alignment of the MACK amino acid sequence with other members of the CC chemokine family. Of significance, the identification of cytokines in human milk is of great interest and is a topic which has been recently
15 investigated (Srivastava et al., Res. Commun. Molec. Path. Pharm. 93:263-283 (1996), which is hereby incorporated by reference).

Example 15 -- Specificity of anti-peptide antisera

Rabbit antisera were raised against three regions (underlined type) of the
20 MACK protein sequence (SEQ. ID. No. 1):

MQQRGLAIVA LAVCAALHAS EAILPIASSC CTEVSHHISR RLLERVNMCR
IQRADGDCDL AAVILHVKRX RICVSPHNHT VKQWMKVQAA XKNGKGNVCH
RKKHHGKRNS NRAHOGKHET YGHKTPY

25 The sequence corresponding to amino acids 32-49 of the MACK protein was designated "MACK A" and has an amino acid sequence corresponding to SEQ. ID. No. 3. The sequence corresponding to amino acids 92-107 of the MACK protein was designated "MACK B" and has an amino acid sequence corresponding to SEQ. ID. No. 4.
30 The sequence corresponding to amino acids 109-127 of the MACK protein was designated "MACK C" and has an amino acid sequence corresponding to SEQ. ID. No. 5.

Antisera against their respective peptides demonstrated high titer, up to dilutions of over 100,000. In addition, anti-peptide antisera reacted with a high degree of specificity to their corresponding immunogen.

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To determine if antisera raised against peptides from the deduced protein sequence of the MACK protein recognized the native protein. Western blotting experiments were performed. Inasmuch as the prostate tissue specific protein PSA is found in the secretion of the prostate gland (i.e., seminal fluid), it was suspected that the MACK protein would be detectable in the secretion of the mammary gland. Of interest, when samples of human milk were examined on Western blotting *versus* the anti-MACK peptide antisera, each of 6 specimens was noted to contain an immunoreactive protein of having an experimentally determined weight of approximately 16-17 kDa. This band was not present when control blots were allowed to react with non-immune rabbit sera, suggesting specificity associated with the use of the anti-MACK peptide antisera. This specificity was confirmed using absorption experiments with soluble peptides. Following absorption of the anti-sera with soluble peptides (100 µg per ml of antiserum dilution), the specific immunoreactive band was abrogated (not shown).

15 Example 16 -- Detection of Mammary Associated Chemokine (MACK) in Breast Cancer Sera Using Western Blotting

Aliquots (1.5 µl) of human sera were heated to 100 °C for 15 min in the presence of reducing agent (mercaptoethanol) and denaturant (sodium dodecyl sulfate ("SDS")) and were then subjected to SDS-polyacrylamide gel electrophoresis ("SDS-PAGE") (as described in Laemmli, which is hereby incorporated by reference) in a 15% PAGE gel. After electrophoresis, the separated proteins were transferred to a nitrocellulose membrane (0.2 µm pore) (Towbin et al., Proc. Natl. Acad. Sci. U.S.A., 76:4350-4354 (1979), which is hereby incorporated by reference). Non-specific protein binding sites on the membrane were blocked with a solution containing bovine serum albumin ("BSA") (2% in tris-buffered saline, pH 7.4) for 1 hr. Thereafter, the membrane was allowed to react for 1 hr with a 1/1000 dilution of polyclonal (rabbit) antisera raised against synthetic peptides corresponding to regions of the MACK gene product, as described in Examples 7 and 15. The membrane was washed thrice in tris-buffered saline and developed with avidin-biotin complex reagents (Pierce Chemicals) according to the recommendations of the manufacturer. Specific bands were revealed following the addition of insoluble alkaline phosphatase substrate (BCIP/NBT).

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The results, presented in Table 6, demonstrated the occurrence of two protein bands (one at 20-30 kDa and one at 7-12 kDa) specifically found in sera obtained from patients with breast cancer. Of 31 such specimens examined, 30 sera demonstrated both bands, while one specimen (number 1871) demonstrated the 20-30 kDa band only.

- 5 In comparison, none of 10 serum specimens obtained from patients with lymphoma or with prostatic, ovarian, lung, or colon cancers showed either of the specific bands when allowed to react with the antibodies to MACK. In addition, MACK peptide bands were not seen in sera obtained from 7 normal individuals (Table 6). These results demonstrate that MACK or MACK-associated proteins are found in the circulation of individuals with
- 10 cancer of the breast and that detection of these immunoreactivities can be of diagnostic and/or monitoring value for the disease.

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TABLE 6

Sample ID	Diagnosis	Stage	High Band ¹	Low Band ²
1008	Breast Cancer	unknown	+	+
1869	Breast Cancer	3	+	+
1870	Breast Cancer	3	+	+
1871	Breast Cancer	3	+	-
1872	Breast Cancer	3	+	+
1873	Breast Cancer	3	+	+
1874	Breast Cancer	3	+	+
1875	Breast Cancer	3	+	+
1876	Breast Cancer	3	+	+
1877	Breast Cancer	3	+	+
1878	Breast Cancer	3	+	+
1293	Breast Cancer	unknown	+	+
1294	Breast Cancer	unknown	+	+
1296	Breast Cancer	unknown	+	+
1297	Breast Cancer	unknown	+	+
1298	Breast Cancer	unknown	+	+
1299	Breast Cancer	unknown	+	+
1300	Breast Cancer	unknown	+	+
1301	Breast Cancer	unknown	+	+
1302	Breast Cancer	unknown	+	+
1303	Breast Cancer	unknown	+	+
2694	Breast Cancer	2	+	+
2697	Breast Cancer	2	+	+
2698	Breast Cancer	2	+	+
4681	Breast Cancer	2	+	+
4682	Breast Cancer	2	+	+
4683	Breast Cancer	2	+	+
4684	Breast Cancer	2	+	+
4686	Breast Cancer	2	+	+
4687	Breast Cancer	2	+	+
4688	Breast Cancer	2	+	+
258	Lung Cancer	3	-	-
259	Lung Cancer	2	-	-
469	Lymphoma	unknown	-	-
470	Lymphoma	unknown	-	-
2486	Prostate Cancer	D	-	-
2488	Prostate Cancer	D	-	-
1939	Ovarian Cancer	4	-	-
1940	Ovarian Cancer	4	-	-
1554	Colon Cancer	C2	-	-
1574	Colon Cancer	C2	-	-
1001	Normal		-	-
1002	Normal		-	-
1003	Normal		-	-
1004	Normal		-	-
1005	Normal		-	-
1006	Normal		-	-
1007	Normal		-	-

¹ High MW Band, approx. 20-30 kDa² Low MW Band, approx. 7-12 kDa

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Although the invention has been described in detail for the purpose of illustration, it is understood that such detail is solely for that purpose and variations can be made by those skilled in the art without departing from the spirit and scope of the
5 invention which is defined by the following claims.

WHAT IS CLAIMED:

1. An isolated chemokine which is preferentially expressed in breast tissue or which is detected in breast milk.
- 5 2. An isolated chemokine according to claim 1, wherein said chemokine comprises from about 100 to about 132 amino acids, has a deduced molecular weight of from about 10 to about 16 kDa, and has a deduced isoionic point of from about pH 10.1 to about pH 10.7.
- 10 3. An isolated chemokine according to claim 1, wherein said chemokine comprises from about 105 to about 127 amino acids, has a deduced molecular weight of from about 12 to about 14 kDa, and has a deduced isoionic point of about pH 10.4.
- 15 4. An isolated chemokine according to claim 1 having an amino acid sequence corresponding to SEQ. ID. No. 1.
- 20 5. An isolated chemokine according to claim 1 having an amino acid sequence corresponding to SEQ. ID. No. 2.
6. A peptide comprising an amino acid sequence corresponding to an antigenic portion of an isolated chemokine according to claim 1.
- 25 7. A peptide according to claim 6, wherein the chemokine has an amino acid sequence corresponding to SEQ. ID. No. 1.
8. A peptide according to claim 6 comprising an amino acid sequence corresponding to SEQ. ID. No. 3.
- 30 9. A peptide according to claim 6 comprising an amino acid sequence corresponding to SEQ. ID. No. 4.
10. A peptide according to claim 6 comprising an amino acid sequence corresponding to SEQ. ID. No. 5.
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11. An isolated antibody which recognizes a chemokine according to claim 1.

12. An isolated antibody according to claim 11, wherein the chemokine
5 has an amino acid sequence corresponding to SEQ. ID. No. 1.

13. An isolated antibody which recognizes a peptide according to claim 6.

10 14. An isolated antibody according to claim 13, wherein the peptide has an amino acid sequence corresponding to SEQ. ID. No. 3.

15 15. An isolated antibody according to claim 13, wherein the peptide has an amino acid sequence corresponding to SEQ. ID. No. 4.

16. An isolated antibody according to claim 13, wherein the peptide has an amino acid sequence corresponding to SEQ. ID. No. 5.

17. An isolated nucleic acid molecule encoding a chemokine according
20 to claim 1.

18. An isolated nucleic acid molecule according to claim 17, wherein the chemokine has an amino acid sequence corresponding to SEQ. ID. No. 1.

25 19. An isolated nucleic acid molecule according to claim 17 having a nucleotide sequence corresponding to SEQ. ID. No. 6.

20. An isolated nucleic acid molecule according to claim 17 having a nucleotide sequence corresponding to SEQ. ID. No. 7.

30 21. An isolated nucleic acid molecule which, under stringent conditions, hybridizes to a nucleic acid molecule encoding a chemokine according to claim 1 or to a complement thereof.

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22. An isolated nucleic acid molecule according to claim 21 which, under stringent conditions, preferentially hybridizes to nucleic acid molecules from breast tissue.

5 23. An isolated nucleic acid molecule according to claim 21 which, under stringent conditions, hybridizes to a nucleic acid molecule encoding a chemokine having an amino acid sequence corresponding to SEQ. ID. No. 1 or to a complement thereof.

10 24. An isolated nucleic acid molecule according to claim 21 which, under stringent conditions, hybridizes to a nucleic acid molecule having a nucleotide sequence corresponding to SEQ. ID. No. 6 or to a complement thereof.

15 25. An isolated nucleic acid molecule according to claim 21 which, under stringent conditions, hybridizes to a nucleic acid molecule having a nucleotide sequence corresponding to SEQ. ID. No. 7 or to a complement thereof.

 26. An isolated nucleic acid molecule according to claim 21 having a nucleotide sequence corresponding to SEQ. ID. No. 8 or a complement thereof.

20 27. An isolated nucleic acid molecule according to claim 21 having a nucleotide sequence corresponding to SEQ. ID. No. 9 or a complement thereof.

 28. An isolated nucleic acid molecule according to claim 21 having a
25 nucleotide sequence corresponding to SEQ. ID. No. 10 or a complement thereof.

 29. An isolated nucleic acid molecule according to claim 21 having a nucleotide sequence corresponding to SEQ. ID. No. 11 or a complement thereof.

30 30. A method for detecting breast disease in a patient comprising:
 contacting a sample of tissue or body fluid from the patient with a
nucleic acid primer which, under stringent conditions, hybridizes to a nucleic acid
molecule encoding a chemokine according to claim 1 or to a complement thereof;

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treating the sample of tissue or body fluid from the patient in contact with the nucleic acid primer under conditions effective to amplify breast tissue specific nucleic acid molecules; and

detecting the breast tissue specific nucleic acid molecules.

5

31. A method according to claim 30, wherein the sample of tissue or body fluid is a blood specimen.

10 32. A method according to claim 30, wherein the sample of tissue or body fluid is a tissue specimen.

33. A method according to claim 30, wherein said detecting the breast tissue specific nucleic acid molecules is carried out by electrophoresis, DNA sequencing, blotting, hybridization, or microscopic visualization.

15

34. A method according to claim 30, wherein said treating is carried out by polymerase chain reaction, ligase chain reaction, self-sustained sequence replication, Q-beta replicase, nucleic acid sequence based amplification, transcription-based amplification system, or branched-DNA methods.

20

35. A method according to claim 30, wherein the nucleic acid primer, under stringent conditions, preferentially hybridizes to nucleic acid molecules from breast tissue.

25

36. A method according to claim 30, wherein the nucleic acid primer, under stringent conditions, hybridizes to a nucleic acid molecule encoding a chemokine having an amino acid sequence corresponding to SEQ. ID. No. 1 or to a complement thereof.

30

37. A method according to claim 30, wherein the nucleic acid primer, under stringent conditions, hybridizes to a nucleic acid molecule having a nucleotide sequence corresponding to SEQ. ID. No. 6 or to a complement thereof.

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38. A method according to claim 30, wherein the nucleic acid primer, under stringent conditions, hybridizes to a nucleic acid molecule having a nucleotide sequence corresponding to SEQ. ID. No. 7 or to a complement thereof.

5 39. A method according to claim 30, wherein the nucleic acid primer, under stringent conditions, hybridizes to a nucleic acid molecule having a nucleotide sequence corresponding to SEQ. ID. No. 8 or to a complement thereof.

40. A method according to claim 30, wherein the nucleic acid primer
10 has a nucleotide sequence corresponding to SEQ. ID. No. 9.

41. A method according to claim 30, wherein the nucleic acid primer has a nucleic acid sequence corresponding to SEQ. ID. No. 10.

15 42. A method of detecting breast disease in a patient comprising:
contacting a sample of tissue or body fluid from the patient with a nucleic acid probe which, under stringent conditions, hybridizes to a nucleic acid molecule encoding a chemokine according to claim 1 or to a complement thereof under conditions effective to permit formation of a hybridization complex between the probe
20 and breast tissue specific nucleic acid molecules; and
detecting the hybridization complex.

43. A method according to claim 42, wherein the sample of tissue or body fluid is a blood specimen.

25 44. A method according to claim 42, wherein the sample of tissue or body fluid is a tissue specimen.

45. A method according to claim 42, wherein said detecting the breast
30 tissue specific nucleic acid molecules is carried out by electrophoresis, DNA sequencing, blotting, microplate hybridization, or microscopic visualization.

46. A method according to claim 42, wherein the nucleic acid probe, under stringent conditions, preferentially hybridizes to nucleic acid molecules from breast
35 tissue.

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47. A method according to claim 42, wherein the nucleic acid probe,
under stringent conditions, hybridizes to a nucleic acid molecule encoding a chemokine
having an amino acid sequence corresponding to SEQ. ID. No. 1 or to a complement
5 thereof.

48. A method according to claim 42, wherein the nucleic acid probe,
under stringent conditions, hybridizes to a nucleic acid molecule having a nucleotide
sequence corresponding to SEQ. ID. No. 6 or to a complement thereof.

10 49. A method according to claim 42, wherein the nucleic acid probe,
under stringent conditions, hybridizes to a nucleic acid molecule having a nucleotide
sequence corresponding to SEQ. ID. No. 7 or to a complement thereof.

15 50. A method according to claim 42, wherein the nucleic acid probe,
under stringent conditions, hybridizes to a nucleic acid molecule having a nucleotide
sequence corresponding to SEQ. ID. No. 8 or to a complement thereof.

20 51. A method according to claim 50, wherein the nucleic acid probe
has a nucleotide sequence corresponding to SEQ. ID. No. 8.

52. A method according to claim 42, wherein the nucleic acid probe
has a nucleic acid sequence corresponding to SEQ. ID. No. 11.

25 53. A method according to claim 42, wherein the probe is bound to a
label selected from the group consisting of a fluorescent label, a radioactive label, a
nuclear magnetic resonance active label, a luminescent label, a chromophore label, and a
detectable functional nucleotide sequence.

30 54. A method of detecting breast disease in a patient comprising:
providing an antibody or binding portion thereof which recognizes
a chemokine according to claim 1;
contacting the antibody or binding portion thereof with a liquid or
tissue sample from the patient under conditions effective to permit binding of the
35 antibody or binding portion thereof to the chemokine in the liquid or tissue sample; and

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detecting presence of the chemokine in the liquid or tissue sample by detecting the binding of the antibody or binding portion thereof to the chemokine in the liquid or tissue sample.

5 55. A method according to claim 54, wherein the antibody or binding portion thereof is bound to a label effective to permit detection of the chemokine upon binding of the antibody or binding portion thereof to the chemokine and wherein said detecting comprises:

 detecting the label.

10

 56. A method according to claim 55, wherein the label is selected from the group consisting of a fluorescent label, a radioactive label, a nuclear magnetic resonance active label, a luminescent label, and a chromophore label.

15

 57. A method according to claim 55, wherein said contacting is carried out in a living mammal and comprises:

 administering the antibody or binding portion thereof to the mammal under conditions effective to permit binding of the antibody or binding portion thereof to the chemokine in the mammal.

20

 58. A method according to claim 57, wherein said administering is carried out orally, parenterally, subcutaneously, intravenously, intramuscularly, intraperitoneally, by intravascular instillation, by intracavitary or intravesical instillation, intraocularly, intraarterially, intralesionally, or by application to mucous membranes.

25

 59. A method according to claim 54, wherein an antibody is used in carrying out said method, said antibody being selected from the group consisting of a monoclonal antibody and a polyclonal antibody.

30

 60. A method according to claim 54, wherein a binding portion is used in carrying out said method, the binding portion being selected from the group consisting of an Fab fragment, an F(ab')₂ fragment, and an Fv fragment.

- 60 -

61. A method according to claim 54, wherein the antibody or binding portion thereof recognizes a chemokine having an amino acid sequence corresponding to SEQ. ID. No. 1

5 62. A method according to claim 54, wherein the antibody or binding portion thereof recognizes a peptide having an amino acid sequence corresponding to SEQ. ID. No. 3.

63. A method according to claim 54, wherein the antibody or binding
10 portion thereof recognizes a peptide having an amino acid sequence corresponding to SEQ. ID. No. 4.

64. A method according to claim 54, wherein the antibody or binding
15 portion thereof recognizes a peptide having an amino acid sequence corresponding to SEQ. ID. No. 5.

65. A method of treating breast disease in a patient comprising:
administering to the patient an effective amount of an antibody or
binding portion thereof which recognizes a chemokine according to claim 1.

20 66. A method according to claim 65, wherein the antibody or binding portion thereof recognizes a chemokine having an amino acid sequence corresponding to SEQ. ID. No. 1

25 67. A method according to claim 65, wherein the antibody or binding portion thereof recognizes a peptide having an amino acid sequence corresponding to SEQ. ID. No. 3.

68. A method according to claim 65, wherein the antibody or binding
30 portion thereof recognizes a peptide having an amino acid sequence corresponding to SEQ. ID. No. 4.

69. A method according to claim 65, wherein the antibody or binding
portion thereof recognizes a peptide having an amino acid sequence corresponding to
35 SEQ. ID. No. 5.

- 61 -

70. A method of treating breast disease in a patient comprising:
administering to the patient an effective amount of a peptide which
binds to a cellular receptor for a chemokine according to claim 1.
- 5 71. A method of vaccinating a patient against breast disease
comprising:
administering to the patient an effective amount of an antigenic
portion of a chemokine according to claim 1.

1/1

Alignment of the MACK amino acid sequence with other members of the CC chemokine family

MACK	MOQRGLAIVA	LAVCA---AL	HASEAILPIA	SS-CCTEVSHH	ISRRLLERV	NMCRIQRADG	DCDLAAVILH	VKRXR--ICV
HTECK	M---NLMLLA	CLVAGFLGAW	APAVHTQGVF	ED-CCLAYHYP	IGNAVLRRA	WTYRIQEVSG	SCNLPAAIIFY	LPKRHRKVCG
LARC	MCCTKSLLLA	ALMSVLL--L	HLCGESEASN	FD-CCLGY--T	DRILHPKFI	VGFTROLANE	GCDINAIIF-	HTKKKLSVCA
TARC	MAPKMLALVT	LLLGASL--Q	HIHAARGTNV	GRECCLEY--F	KGAIPLRKL	KTWYQ--TSE	DCSRDAIVF-	VTVQGRAICS
I309	MQIITTALVCLLLAG	M-----WP--E	DVDSKSMQVP	FSRCCFSF--A	EQEIPLRAI	LCY-R-NTSS	ICSNEGLIF-	KLKRGEACA
MCP-2			AQPDVSIS	PITCCFNV--I	NRKIPQRL	ESYTR-ITNI	QCPKEAVIF-	KTKRGKEVCA
MCP-4			GLAQPDALNV	PSTCCFTF--S	SKKISLQRL	KSYV--ITTS	RCPOKAVIF-	RTKLKGKEICA
Eotaxin			GLAGPASV--	PTTCCFNL--A	NRKIPLQRL	ESYRR-ITSG	KCPQKAVIF-	KTKLAKDICA
MCP-3			GLAQPVGINT	STTCCYRF--I	NKKIPKQRL	ESYRR-ITSS	HCPREAVIF-	KTKLDKEDCA
MCP-1			GLAQPDAINA	PVTCCYNF--T	NRKISVQRL	ASYRR-ITSS	KCPKEAVIF-	KTIVAKEDCA
RANTES			ASASPYSSDT	-TPCCFAY--I	ARPLPRAHI	KEYFY--TSG	KCSNPVAVF-	VTRKNRQVCA
HCC-1			TESSSRGPYH	PSECCFTY--T	TYKIPRQRI	MDIYE--TNS	QCSKPGIVF-	ITKRGHVSCT
MIP-1B			ALSAPMGSDP	PTACCFSY--T	ARKLPNRFV	VDIYE--TSS	LCSQPAVVF-	QTKRSKQVCA
LB78B			VLSAPLAADT	PTACCFSY--T	SRQIPQNF	ADYFE--TSS	QCSKPSVIF-	LTKRGRQVCA
LD78A			-FSASLAADT	PTACCFSY--T	SRQIPQNF	ADYFE--TSS	QCSKPGVIF-	LTKRSRQVCA
PARC			--SCAQVGTN	KELCCLVY--T	SWQIPQKFI	VDYSE--TSP	QCPKPGVIL-	LTKRGRQDCA
MACK	SPHNHTVKQW	MKVQAAXKNG	KGNVCHRRK-	-----HHGKR	NSNRAHQKH	ETYGHKTP--	-----	---Y----
HTECK	NPKSREVQRA	MKLLDARNKV	FAKLHHNMOT	FOAGPHAVKK	LSSGNSKLSS	SKFSNPISSS	KRNVSLISA	NSGL----
LARC	NPKQTMV-KY	I-----	-----	-----	-----	-----	---VRLSSKK	VKNM----
TARC	DPNNQRV-KN	A-----	-----	-----	-----	-----	---VKYLQSL	ERS----
I309	LDTVGMV-OR	H-----	-----	-----	-----	-----	---RQMLRHC	PSKRK--
MCP-2	DPKERWV-RD	S-----	-----	-----	-----	-----	---MKHLDQI	FQNLKP--
MCP-4	DPKEKVV-QN	Y-----	-----	-----	-----	-----	---MKHLGRK	AHTLKT--
Eotaxin	DPKKKWV-QD	S-----	-----	-----	-----	-----	---MKYLDQK	SPTPKP--
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MIP-1B	DPSEWV-QE	Y-----	-----	-----	-----	-----	---VYDLELN	
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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/00651

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/19 C07K14/52 A61K38/19 A61K39/395 G01N33/53
 C12Q1/68 C07K16/24

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K G01N C12Q A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 98 23750 A (SCHERING CORPORATION (US); VICARI A; MORALES J; HEDRICK J.A; ZLOTNIK A) 4 June 1998 see abstract Seq.ID:1,2 see page 58 - page 60 ---	1-13, 17-25, 29-38, 42-49, 52-61, 65,66, 70,71
E	WO 99 06439 A (GENSET (FR); DUMAS MILNE EDWARDS J.-B.; DUCLERT A.; LACROIX B.) 11 February 1999 see abstract Seq.ID:163 see page 136 - page 137 ---	4-7,11, 12, 18-21, 23-25,29
-/--		

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"Z" document member of the same patent family

Date of the actual completion of the international search

13 April 1999

Date of mailing of the international search report

05.05.99

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
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 Fax: (+31-70) 340-3016

Authorized officer

Macchia, G

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 99/00651

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Remarks: Although claims 54,55,56,59-64 (as far as in vivo methods are concerned), and 57,58 are directed to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition. Although claims 65-71 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.